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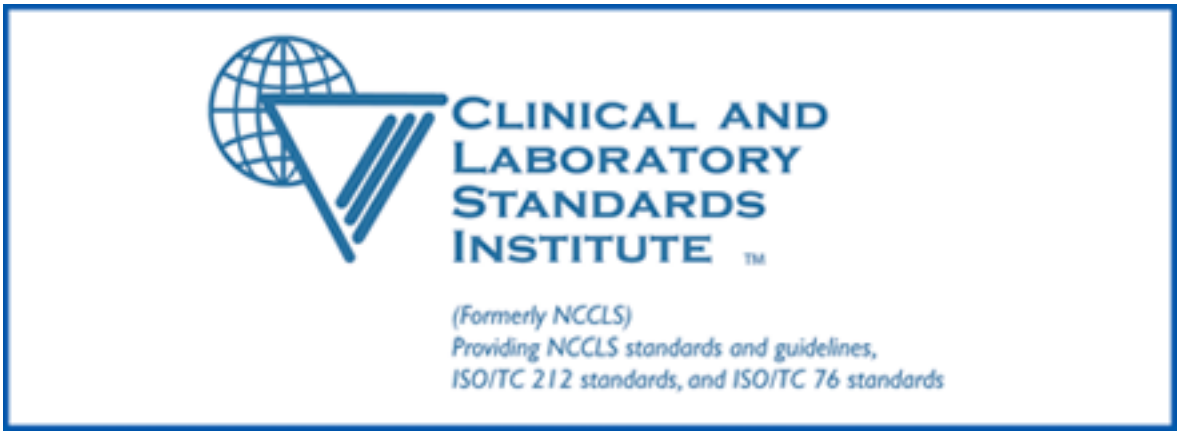
Susceptibility Testing of Mycobacteria, Nocardiae, and Other Aerobic Actinomycetes; Approved Standard



This standard provides protocols and related quality control parameters and interpretive criteria for the susceptibility testing of mycobacteria, *Nocardia* spp., and other aerobic actinomycetes.

A standard for global application developed through the NCCLS consensus process.

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Susceptibility Testing of Mycobacteria, Nocardiae, and Other Aerobic Actinomycetes; Approved Standard

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Abstract

This document addresses the susceptibility testing of *Mycobacterium tuberculosis* complex (MTBC), clinically significant slowly and rapidly growing mycobacterial species, *Nocardia* spp., and other aerobic actinomycetes. Included in this standard are recommendations for the selection of agents for primary and secondary testing, organism group-specific methodologies, reporting recommendations, and quality control criteria for the above-listed organisms. Recommendations regarding the selection of agents for testing mycobacteria are based primarily on guidelines from U.S. agencies. For testing MTBC, M24-A recognizes the method of agar proportion as the primary methodology upon which all other methodologies are essentially based; there are also recommendations for use of commercial broth susceptibility methods with shorter incubation times, which are now in widespread use in the susceptibility testing of this significant group of microorganisms.

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Foreword

NCCLS document M24-A addresses *Mycobacterium tuberculosis* complex (MTBC), certain nontuberculous mycobacteria (NTM), and in this version of the document, information regarding *Nocardia* and other aerobic actinomycetes is presented for the first time. The breakpoints for *Nocardia* and other aerobic actinomycetes are based on PK/PD data, organism population distributions, clinical data, breakpoints used for other organisms, and the experience of experts in the field. Currently, sufficient data exist to support susceptibility testing recommendations for MTBC and tentative recommendations for *Mycobacterium avium* complex, *Mycobacterium kanasii,* the rapidly growing mycobacteria (*Mycobacterium fortuitum* group, *Mycobacterium abscessus,* and *Mycobacterium chelonae*), and aerobic actinomycetes.

Laboratory tests for evaluating the susceptibility of mycobacteria and aerobic actinomycetes can confirm the choice of the initial course of chemotherapy, and they can confirm the emergence of drug resistance when a patient fails to show a satisfactory bacteriologic response to treatment, as well as guide the choice of further treatment with different drugs. Susceptibility testing of MTBC can also be used to estimate the prevalence of primary and acquired drug resistance (defined by the World Health Organization as "drug resistance among new cases" and "drug resistance among previously treated patients"[1]) in a community. For each of these purposes, use of a reliable technique to perform the test is essential.

For MTBC, susceptibility testing should be performed on the initial isolate from all patients. Susceptibility testing should be repeated if the patient is culture-positive after three months of appropriate therapy or shows clinical evidence of failure to respond to therapy. To assure the earliest possible detection of resistance, a commercial, shorter incubation system should be used in conjunction with rapid methods for primary culture and identification. In this way, susceptibility test results for most isolates should be reported within 15 to 30 days of receipt of the specimen in the laboratory.

In contrast to MTBC, susceptibility testing of NTM and aerobic actinomycetes should be performed on the initial isolate only for clinically significant isolates that exhibit variability in susceptibility to clinically useful antimicrobial agents and/or significant risk of acquired mutational resistance to one or more of these agents. Because the latter two criteria are not true for *Mycobacterium marinum*, routine susceptibility testing of this species is not recommended.

To determine clinical significance of NTM recovered from respiratory cultures, the American Thoracic Society currently recommends the following criteria: three positive cultures with negative smears for acid-fast bacilli (AFB) or two positive cultures and one positive smear are usually sufficient to confirm clinical significance. Alternatively, if only one bronchial wash is available and it is culture-positive or the AFB smear is ≥2+, this is sufficient to establish clinical significance. In addition, isolates from normally sterile sites (such as blood, cerebrospinal fluid, or tissues) typically are considered clinically significant.

All of the authors of this document donated considerable time to its development; I would like to personally thank all of them for their valuable contributions.

Gail L. Woods, M.D.
Chairholder, Subcommittee on Antimycobacterial Susceptibility Testing

In an effort to increase the clinical utility of the M24 standard, the Subcommittee on Antimycobacterial Susceptibility Testing opted not only to revise the recommendations for testing of MTBC, but also to present susceptibility testing methodologies for nontuberculous mycobacteria and the aerobic actinomycetes.

Standard Precautions

Because it is often impossible to know what might be infectious, all human specimens are to be treated as infectious and handled according to "standard precautions." Standard precautions are new guidelines that combine the major features of "universal precautions and body substance isolation" practices. Standard precautions cover the transmission of any pathogen and thus are more comprehensive than universal precautions which are intended to apply only to transmission of blood-borne pathogens. Standard precaution and universal precaution guidelines are available from the U.S. Centers for Disease Control and Prevention (*Guideline for Isolation Precautions in Hospitals*. Infection Control and Hospital Epidemiology. CDC. 1996;Vol 17;1:53-80), (MMWR 1987;36[suppl 2S]2S-18S), and (MMWR 1988;37:377-382, 387-388). For specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials and for recommendations for the management of blood-borne exposure, refer to the most current edition of NCCLS document M29—*Protection of Laboratory Workers from Occupationally Acquired Infections.*

The mycobacteriology laboratory presents a unique set of circumstances in terms of observance of biosafety precautions. For more information, it is suggested that the reader refer to: *Clinics in Laboratory Medicine, 1996: Biosafety in the Clinical Mycobacteriology Laboratory*[2] and the 1999 U.S. government publication *Biosafety in Microbiological and Biomedical Laboratories*.[3]

A Note on Terminology

NCCLS, as a global leader in standardization, is firmly committed to achieving global harmonization wherever possible. Harmonization is a process of recognizing, understanding, and explaining differences while taking steps to achieve worldwide uniformity. NCCLS recognizes that medical conventions in the global metrological community have evolved differently in the United States, Europe, and elsewhere; that these differences are reflected in NCCLS, International Organization for Standardization (ISO), and European Committee for Standardization (CEN) documents; and that legally required use of terms, regional usage, and different consensus timelines are all obstacles to harmonization. In light of this, NCCLS recognizes that harmonization of terms facilitates the global application of standards and is an area of immediate attention. Implementation of this policy must be an evolutionary and educational process that begins with new projects and revisions of existing documents.

Of particular note in M24-A, are two terms whereby NCCLS intends to eliminate confusion, over time, through its commitment to harmonization. For the most part, in this guideline, the term "accuracy" is used correctly in its metrological sense, to refer to the closeness of the agreement between the result of a (single) measurement and a true value of a measurand, thus comprising both random and systematic effects. But there are several instances in this document, where accuracy is defined the way ISO defines "trueness," i.e., the closeness of the agreement between the average value from a large series of measurements and to a true value of a measurand. To facilitate understanding, when used this way, "trueness" has been inserted parenthetically. Also, the terms are defined in the guideline's Definitions section along with explanatory notes. During the next scheduled revision of this document, they will be reviewed for consistency with international use, and revised appropriately.

Key Words

Antimycobacterial drugs, antituberculous drugs, drug susceptibility

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1 Scope

This document contains protocols for the susceptibility testing of three major categories of mycobacterial species: 1) *Mycobacterium tuberculosis* complex (MTBC), 2) the slowly growing, nontuberculous mycobacteria (NTM), and 3) the rapidly growing mycobacteria. Additionally, recommendations for susceptibility testing of *Nocardia* spp. and miscellaneous aerobic actinomycetes are included in this document. M24-A contains guidance on the selection of primary and secondary agents for testing and reporting; instructions for performing the standard agar proportion method and other reference methods (e.g., broth micro- and macrodilution for *Mycobacterium avium* complex and broth microdilution for rapidly growing mycobacteria); and quality control protocols for each organism category. Testing and reporting recommendations and principles of quality control procedures apply to using commercial FDA-cleared systems as well as the reference methods. It is anticipated that further refinement of these protocols will occur as laboratories involved in the regular testing of these organism types gain experience with the use of this document. To facilitate further development of M24, the subcommittee requests comments and suggestions for improvement with regard to the methods included herein.

2 Definitions

Accuracy - Closeness of the agreement between the result of a measurement and a true value of the measurand (VIM93-3.5.)[4] **NOTE:** There are several occasions in this document where accuracy is defined the way ISO defines **Trueness,** defined below. When used in this way, the term "trueness" has been inserted parenthetically.

Antimicrobial Susceptibility Test Interpretive Category – A classification based on an *in vitro* response of an organism to an antimicrobial agent; **NOTES:** a) For mycobacteria two different categories, "critical concentration" and "minimum inhibitory concentration" have been used to categorize the *in vitro* results; b) For members of the MTBC, when tested against the lower concentration of some agents, the "critical concentration" category is applied. Testing of an additional higher concentration may also be recommended for some agents. However, there is no "intermediate" interpretive category when the "critical concentration" category is applied, even when testing is performed both at the critical concentration and the additional higher concentration; c) For NTM and for the aerobic actinomycetes, only the "minimum inhibitory concentration" category is applied.

Borderline Antimicrobial Susceptibility Test Interpretive Category – An interpretive category applicable only to certain results obtained with MTBC isolates tested against pyrazinamide by the radiometric instrument method; **NOTE:** Repeat testing may resolve whether the isolate in question is susceptible or resistant.

Critical concentration – The "critical concentrations" of antituberculous drugs were adopted by international convention;[5] **NOTE:** For each drug, the critical concentration is the lowest concentration that inhibits 95% of "wild-type" strains of *M. tuberculosis* that have not been exposed to the drug, but that simultaneously does not inhibit strains of *M. tuberculosis* considered resistant that are isolated from patients who are not responding to therapy.

Culture – **1)** The intentional growing of microorganisms, such as bacteria, viruses, or tissues, in a controlled environment, for purposes of identification or other scientific study, or for commercial and/or medicinal use; **2)** The product resulting from the intentional growth of microorganisms or tissue.

Culture medium – A substance or preparation used for the cultivation and growth of microorganisms or tissue.

Intermediate Antimicrobial Susceptibility Test Interpretive Category – For ***Minimal inhibitory concentration***, an interpretive category that implies that an infection due to the isolate may be appropriately treated in body sites where the drugs are physiologically concentrated or when a high dosage of drug can be used; also indicates a "buffer zone" that should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations.

Measurand - Particular quantity subject to measurement [VIM93-2.6].[4]

Minimal inhibitory concentration, MIC – The lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in an agar or broth dilution susceptibility test.

***Mycobacterium avium* complex, MAC** – Includes the species *M. avium* and *M. intracellulare*, and often an additional organism not yet formally named that is sometimes referred to as the "X"-cluster.

***Mycobacterium tuberculosis* complex, MTBC** – Includes the species *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, and *M. canettii*.

Nontuberculous mycobacteria, NTM – All species of mycobacteria other than those in the MTBC.

Resistant Antimicrobial Susceptibility Test Interpretive Category – 1) For ***Critical concentration,*** Resistance is defined as diminished susceptibility of a strain that differs from wild-type strains from patients who have not been treated with the drug, so that the strain is unlikely to show clinical responsiveness to the drug; 2) For ***Minimal inhibitory concentration,*** resistant isolates are not inhibited by the usually achievable concentrations of the agent at the site of infection with normal dosage schedules, and/or fall in the range where specific microbial resistance mechanisms are likely (e.g., beta-lactamases), and clinical efficacy has not been reliable in treatment studies.

Susceptible Antimicrobial Susceptibility Test Interpretive Category – **1)** For ***Critical concentration*****,** a level of susceptibility that is not significantly different from that of wild-type strains from patients who have not been treated with the drug, so that the strain is likely to show clinical responsiveness to the drug; 2) For ***Minimal inhibitory concentration*****,** an interpretive category that implies that an infection due to the isolate may be appropriately treated with the dosage of an antimicrobial agent recommended for that type of infection and infecting species, unless otherwise indicated, given the levels attainable with the agent at the site of infection.

Trueness - The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value (ISO 3534-1).[6]

Validation - Confirmation through the provision of objective evidence, that requirements for a specific intended use or application have been fulfilled; (ISO 9000:2000 [3.85]).[7]

Verification - Confirmation through the provision of objective evidence that specified requirements have been fulfilled (ISO 9000:2000 [3.8.4]).[7]

3 Antimycobacterial Susceptibility Testing for *Mycobacterium tuberculosis* Complex

3.1 Introduction

Current methods for susceptibility testing of *M. tuberculosis* complex (MTBC, i.e., *M. tuberculosis*, *M. bovis, M. africanum, M. microti, M. canettii*) are based on proportion methods and are considered equivalent to the standard methods established by Canetti et al.[5] The proportion methods used globally rely on a bacteriological definition of drug resistance that was developed in recognition of the difficulties in defining clinical resistance for mycobacteria. Resistance is defined as "a decrease in sensitivity of sufficient degree to be reasonably certain that the strain concerned is different from a sample of wild strains of human type that have never come into contact with the drug."[8] For several decades the method of proportion using Middlebrook 7H10 agar[9] has been considered the standard method in the United States and is described in this document.[a]

The agar proportion and radiometric[a] methods both define resistance as growth of greater than 1% of an inoculum of bacterial cells in the presence of a "critical" concentration of antituberculous drug. The critical concentrations of antituberculous drugs were adopted by international convention and represent the lowest concentrations of drugs that inhibit 95% of "wild strains" of *M. tuberculosis* that have never been exposed to the drugs, while at the same time not inhibiting strains of *M. tuberculosis* that have been isolated from patients who are not responding to therapy, and that are considered resistant. The recommended critical concentrations of drug were originally determined in egg-based Lowenstein-Jensen medium, and equivalent concentrations of drugs were later established in Middlebrook 7H10 and 7H11 for the agar proportion method[10] and in the media used in commercial susceptibility test systems.[11-13] Every laboratory should test the susceptibility of MTBC to the critical concentration of drug for the test method it is using. The critical concentration is the standard that allows interpretation of tests by any of the procedures. When greater than 1% of the tested bacterial population in a clinical isolate becomes resistant to the critical concentration of a drug, that drug is not (or soon will not be) useful for continued antituberculous chemotherapy.[9]

Using the critical concentrations of primary antituberculous drugs (i.e., isoniazid [INH], rifampin [RIF], ethambutol [EMB], and pyrazinamide [PZA]), the results of *in vitro* susceptibility testing of these correlate well with clinical effectiveness in patients with tuberculosis. Data concerning testing of secondary antituberculous drugs (see Table 1), however, are more limited.

Although the agar proportion and commercial broth susceptibility methods represent breakpoint susceptibility testing using a single, critical concentration of drug, laboratories may test an additional higher concentration of INH. The critical concentration of drug determines whether the isolate is considered resistant. The additional higher concentration of INH, however, can provide the physician with information about the level of drug resistance to help make decisions on treatment regimens.

Treatment practices differ among clinicians for INH-resistant MTBC, and there are no randomized clinical trials that would provide the basis for recommendations. Therefore, treatment practices are based primarily on expert opinion. The laboratory's role is to provide the clinician with timely and comprehensive information on the susceptibility to the antituberculous drugs. Testing two concentrations of INH provides an indication of the level of drug resistance, and is endorsed by the Centers for Disease

[a] The radiometric BACTEC 460TB (BD, Sparks, MD) and the nonradiometric BACTEC MGIT 960 (BD) and ESP II System (Trek Diagnostics, Inc., Westlake, OH) are broth methods designed to give results equivalent to the agar proportion method. These three systems are the only ones cleared by the U.S. Food and Drug Administration. Other broth-based susceptibility testing methods are in development. These systems are referred to generically throughout this document as "rapid broth" methods.

Control and Prevention's (CDC) Advisory Committee for Elimination of TB. If only one concentration of INH is tested, it should be the lower, critical concentration of drug.

When an isolate of MTBC is tested against two concentrations of INH, and is resistant to the lower concentration but susceptible to the higher concentration, the following comment should be appended to the results: "These test results indicate low-level resistance to INH. Some experts believe that patients infected with strains exhibiting this level of INH resistance may benefit from continuing therapy with INH. A specialist in the treatment of tuberculosis should be consulted concerning the appropriate therapeutic regimen and dosages."

Users of this document should be aware that the standardized agar proportion method for susceptibility testing of MTBC described here is not a rapid method. To assure the earliest possible detection of resistant organisms, a broth method with a shorter incubation time is the recommended standard of practice for drug susceptibility testing of MTBC in the United States and many industrialized nations.[14] Using a commercial broth susceptibility testing method with a shorter incubation time, in conjunction with primary culture and identification approaches that reduce lengthy incubations, enables earlier reporting of results. The CDC has recommended that mycobacteriology laboratories work toward the goal of reporting first-line susceptibility results for MTBC within 15 to 30 days of receipt of the initial diagnostic specimen.[15] Ideally, susceptibility results should be available within 7 to 14 days after isolation of MTBC. In reporting susceptibilities, local and state regulations should be followed by the reporting laboratory.

Procedural instructions in this document for performing the reference agar proportion method or other reference methods (e.g., broth macro- and microdilution) do not apply to commercial broth systems. Manufacturers of those systems determine unique drug concentrations and specific procedural instructions to produce results equivalent to the agar proportion method. Each laboratory using these systems is responsible for following those specific procedural instructions. If changes are made to the manufacturer's procedure, laboratories are required under CLIA regulations to validate performance. Commercial system manufacturers are obligated to ensure that system performance meets user needs and performs reliably when testing is done in accordance with the product labeling.

At this time, there are three FDA-cleared broth systems for indirect testing of *M. tuberculosis*. These are recommended in this document for initial susceptibility testing with primary antituberculous drugs. There have been no commercially available broth systems cleared by FDA for slow growing nontuberculous mycobacteria, rapid growing mycobacteria, actinomycetes or nocardia. The agar proportion method on the other hand is used to confirm results from commercial broth systems, to test additional drugs or concentrations of drugs that are not available in those commercial systems. The agar proportion method is also used as the standard against which new methods are evaluated and for characterizing *in vitro* susceptibility of new antimycobacterial drugs.

The first isolate of MTBC obtained from every patient should be tested.[14,16] Susceptibility testing should be repeated if there is clinical evidence of failure to respond to therapy, or if cultures fail to convert to negative after three months of therapy. Any commercial shorter-incubation system utilized should have been previously demonstrated to produce results that correlate with those obtained with the standardized agar proportion method. If the results obtained for a patient's isolate tested against any agent, by any commercial shorter-incubation system, indicate resistance, or if the results, by any commercial shorter-incubation system, are in any way ambiguous or problematic, then immediate repeat testing of the isolate against that agent using the standardized agar proportion method may be warranted. **A report of this initial result should not be delayed while repeat testing is being performed. This report should indicate that the drug resistance findings are preliminary and confirmatory testing has been initiated.** Consideration should be given to testing of secondary agents at the same time as any repeat testing, so that several drugs to which the isolate is susceptible can be identified and results for secondary agents are not delayed.

The full panel of primary drugs for susceptibility testing of MTBC includes INH at two concentrations (critical and higher concentration), RIF, EMB, and PZA. This represents a combination of tests that provides the clinician with comprehensive information related to the four-drug therapy currently recommended for treatment of most patients in the United States with tuberculosis.[16] Including PZA and a higher concentration of INH in the panel provides immediate additional information about the efficacy of four-drug therapy when resistance is encountered. The full panel of primary drugs may also provide sufficient information to avoid unnecessary secondary drug testing when a strain of MTBC is resistant only to INH, which is the most frequent pattern in the United States.[17] Drug susceptibility testing with commercial shorter-incubation systems, however, is expensive, requiring that laboratories make decisions about cost-effective testing. Laboratory directors should consult with their pulmonary and/or infectious disease specialist and TB control officer when making decisions concerning reducing the number of drugs tested. The decision to test fewer or more (e.g., including streptomycin) drugs should be based on considerations of: 1) the patient population served; 2) prevalence of drug resistance; 3) standard drugs used for treatment within the community; and 4) the availability and timeliness of obtaining additional testing when resistance or drug intolerance is encountered. In many areas, for example, laboratories may consider testing a single, critical concentration of INH, RIF, and EMB only. State, provincial, and local public health laboratories serve as referral centers for mycobacterial testing, including drug susceptibility testing for MTBC. At a minimum, state and provincial public health laboratories should provide, or assure access to, the full panel of primary antituberculous drugs and secondary drugs. This reference service is necessary to provide continued surveillance of drug resistance and to rapidly augment testing for laboratories that may choose to test less than the full panel of primary drugs.

Whenever secondary drug testing is required, laboratories should avoid a "piecemeal" approach to providing clinicians with additional drug susceptibility test results. This is a particular concern, because currently most secondary drug testing is performed using the slower agar proportion method. If a laboratory routinely tests only the lower, critical concentration of INH (in addition to other primary drugs), any isolate of MTBC that is resistant to that concentration should also be tested to the higher concentration of INH. Whenever an isolate of MTBC is resistant to RIF or resistant to any two of the primary drugs, all secondary drugs (listed in Table 1) and a higher concentration of EMB should also be tested. If such testing is not done in-house, the isolate should immediately be forwarded to a public health or other referral laboratory. Isolates should be retained until results are available to complete reporting requirements. Additionally, for patients with resistant isolates, including resistance only to the lower, critical concentration of INH, referral to or consultation with a specialist in tuberculosis treatment should be considered.

3.2 Agar Proportion Method

This procedure is performed by inoculating equal quantities of several dilutions of a standardized inoculum onto agar-based medium with and without the test drug. Separate, countable colonies should be observed on a control quadrant without any of the drug. The number of colony forming units (CFU) growing on the drug-containing medium compared with those growing on the drug-free medium are then determined and expressed as a percentage. Strains of tubercle bacilli in which growth on drug-containing media represents more than 1% of the number of colonies that develop on drug-free media are considered to be resistant to that agent.[9] The agar proportion method using Middlebrook 7H10 agar medium is recommended by the U.S. Public Health Service.

3.2.1 Antituberculous Agents

3.2.1.1 Source

Antimicrobial standards or reference powders, for use with the agar proportion method, can be obtained from commercial sources. Most antimicrobial reference powders are also available from:

U.S. Pharmacopoeial Convention, Inc.
Reference Standards Order Department
12601 Twinbrook Parkway
Rockville, MD 20852, USA

Pharmacy stock or other clinical preparations are not to be used. Acceptable drug standards bear a label that states the generic name, its assay potency [usually expressed in micrograms (μg) of drug per mg of powder], and its expiration date. The antimicrobial powders are to be stored as recommended by the manufacturer or at -20 ^{o}C or below in a desiccator (preferably in a vacuum). When the desiccator is removed from the freezer, it is to come to room temperature before it is opened (to avoid condensation of water).

3.2.1.2 Weighing Antituberculous Drugs

All antimicrobial agents are assayed for standard units of activity. The assay units may differ widely from the actual weight of the powder and often may differ between drug production lots. Thus, a laboratory must standardize its antimicrobial solutions based on assays of the lots of antimicrobial powders that are being used.

The value for potency supplied by the manufacturer should include consideration of measures of purity (usually by high-performance liquid chromatography (HPLC) assay), water content (e.g., by Karl Fischer analysis or by weight loss on drying), and the salt/counter-ion fraction (if the compound is supplied as a salt instead of free acid or base). The potency may be expressed as a percentage, or in units of μg/mg (w/w).

In some cases, a certificate of analysis with values for each of these components may be provided with antibiotic powders; in this case, an overall value for potency may not be provided, but can be calculated from HPLC purity, water content, and when applicable, the active fraction for drugs supplied as a salt (e.g., hydrochloride, mesylate). However, if when testing these calculations, any value is unknown or is not clearly determined from the certificate of analysis, it is advisable that the factors used in this calculation be confirmed with the supplier or the manufacturer. The following demonstrates an example calculation:

Example: meropenem trihydrate

Certificate of analysis data:

Assay purity (by HPLC): 99.8%

Measured water content (by Karl Fischer analysis): 12.1% (w/w)

Active fraction: 100% (supplied as the free acid, and not a salt)

Potency calculation from above data:

Potency = (Assay purity) x (Active fraction) x (1- Water Content)

Potency = (0.998) x (1.0) x (1- 0.121)

Potency = 0.877 μg/mg or 87.7%

Either of the following formulas below may be used to determine the amount of powder of diluent needed for a standard solution:

$$\textit{Weight}\ (\text{mg}) = \frac{\textit{Volume}\ (\text{mL}) \bullet \textit{Concentration}\ (\mu\text{g/mL})}{\textit{Potency}\ (\mu\text{g/mg})} \quad (1)$$

or

$$\textit{Volume}\ (\text{mL}) = \frac{\textit{Weight}\ (\text{mg}) \bullet \textit{Potency}\ (\mu\text{g/mg})}{\textit{Concentration}\ (\mu\text{g/mL})} \quad (2)$$

The antimicrobial powder should be weighed on an analytical balance that has been calibrated with National Institute of Standards and Technology (NIST) weights (or other approved reference weights). If possible, more than 100 mg of powder should be weighed. It is advisable to accurately weigh a portion of the antimicrobial agent in excess of that required and to calculate the volume of diluent needed to obtain the final concentration desired as in formula 2 above.

Example: To prepare 100 mL of a stock solution containing 1,280 µg/mL of antimicrobial agent with antimicrobial powder that has a potency of 750 µg/mg, 170 to 200 mg of the antimicrobial powder should be accurately weighed. If the actual weight is 182.6 mg, the volume of diluent needed is then as follows:

$$\textit{Volume}\ (\text{mL}) = \frac{182.6\ \text{mg} \bullet 750\ \mu\text{g/mg}}{(\textit{Actual Weight})\quad(\textit{Potency})} = 107.0\ \text{mL} \quad (3)$$
$$1{,}280\ \mu\text{g/mL}$$
$$(\textit{Desired Concentration})$$

Therefore, the 182.6 mg of antimicrobial powder is to be dissolved in 107.0 mL of diluent.

3.2.1.3 Selection and Concentration of Antituberculous Drugs

The primary antituberculous drugs are INH, RIF, EMB, and PZA. Table 1 lists the concentrations recommended for the first three drugs for use with the agar proportion method, using 7H10 or 7H11 agar medium.[18] See Section 3.4 of this document for a discussion of susceptibility testing of PZA. Testing all of the secondary antituberculous drugs listed in Table 1 should be performed on all isolates of MTBC that are resistant to RIF or resistant to any two of the primary drugs.

3.2.1.4 Preparation and Storage of Stock and Working Solutions

Examples of stock solution concentrations of antituberculous drugs made from drug powders or lyophilized commercial products are noted in Table 2.

3.2.1.4.1 Drug Powders

Stock solutions of antituberculous agents available as powders are to be prepared at concentrations of at least 1,000 µg/mL and preferably 10,000 µg/mL, except as noted in Table 2, footnote †. Approximately 100 mg of drug (depending on the potency) dissolved in 10 mL of sterile distilled water would yield a stock solution of 10,000 µg/mL.

Some drugs must be dissolved in solvents other than water. In such cases it is necessary to only use sufficient solvent to solubilize the antimicrobial powder, and then dilute to the final stock concentration with sterile distilled water or appropriate buffer, as suggested in Table 2.

Sterilize solutions using a membrane filter (e.g., cellulose nitrate or mixed cellulose ester [nitrate and acetate]) with a pore size of 0.22 µm. Paper, asbestos, or sintered glass filters, which may absorb

appreciable amounts of certain antituberculous agents, are not to be used. The first 10 to 15% of the filtered solution is discarded, because initially, some of the drugs could adsorb to the filter.

Small volumes of the sterile stock solutions are dispensed into sterile polypropylene or polyethylene vials appropriate for low-temperature storage, carefully sealed, and stored for up to 12 months at -70 ºC. Thaw to room temperature and use without delay, discard excess, and never refreeze. Lower concentration stock solutions and higher storage temperatures have also demonstrated satisfactory stability for 12 months (i.e., capreomycin 1,000 µg/mL at -20 ºC, streptomycin 2,000 µg/mL at 2 to 8 ºC, and PAS 2,000 µg/mL at 3 to 7 ºC). In all cases, directions provided by the drug manufacturer are considered to be part of these general recommendations.

3.2.2 Preparation of Drug Medium

3.2.2.1 7H10 Agar Medium

7H10 agar medium is recommended for susceptibility testing. 7H11 agar medium, which can be of help in the recovery of INH-resistant strains of MTBC, is an acceptable alternative. Those who do use 7H11 agar should be aware that different concentrations of some antimycobacterial agents must be used with this medium (see Table 1).

These guidelines are restricted to Middlebrook agar media only. Guidelines and drug concentrations for inspissated egg media for susceptibility testing of MTBC are different and can be found in the literature.[19] Egg-based media for susceptibility testing of NTM are not recommended, as these media are either inhibitory or not optimum for growth of many NTM.

7H10 agar medium is prepared from a dehydrated base as recommended by the manufacturer. After the agar is autoclaved, it is allowed to cool to 50 to 56 ^{o}C in a water bath before adding the required oleic acid-albumin-dextrose-catalase (OADC) supplement (warmed to room temperature, 22 to 25 ^{o}C) and the appropriate antimycobacterial agent. This medium is usually prepared in lots of 200 mL.

The antituberculous agent is incorporated into 7H10 agar medium contained in plastic quadrant petri dishes. As a growth control, one quadrant in each plate is filled with 7H10 agar medium without any added drug. The other three quadrants contain different concentrations of one or more of the agents to be tested. There are two methods of preparing a drug medium: agar dilution and disk elution.

There are two methods for preparing the drug medium: 1) drug is delivered to the medium via a disk; and 2) liquid drug is added to the medium and either a disk of the antituberculous agent is placed in the quadrant and 7H10 agar medium is poured over it (method 1) or each of the antituberculous agents is incorporated into 7H10 agar medium and poured into one of three quadrants of a petri dish (method 2). One of the quadrants of the petri dish is filled with 7H10 agar medium that does not contain any antituberculous agents. This quadrant serves as a positive growth control. The other three quadrants contain agents to be tested.

3.2.2.2 Medium with Drug-Containing Disks

Many laboratories use the method of Wayne and Krasnow in which paper disks containing specified amounts of antituberculous drugs are placed in individual quadrants of 100-mm plastic petri plates.[20] Commercially prepared disks (see Table 3 for suggested final concentrations) are available. A 5-mL amount of drug-free 7H10 agar medium is dispensed into each quadrant. This simplified procedure allows each laboratory to make just the number of plates needed for immediate use and eliminates labeling error, because the disks carry identification codes and antimicrobial contents. The reliability of this method has been established and results have been shown to be comparable to those achieved by standard methods.[21,22]

3.2.2.2.1 Preparation of Media with Drug-Containing Disks

In preparing disk elution plates, it is necessary to perform the following steps:

(1) Dispense the disks into individual quadrants of sterile petri dishes using aseptic technique.

(2) Prepare complete OADC-enriched 7H10 agar medium (180 mL of 7H10 agar plus 20 mL OADC, as described in Section 3.2.2.1). Dispense 5.0 mL of this medium into each quadrant, overlaying the disk while keeping it approximately centered in the quadrant. Accurate delivery of 5.0 mL of medium into each quadrant is essential to assure the correct final concentration of drug. Allow the agar to solidify at room temperature.

(3) Before use or storage, plates can be thoroughly dried and the drug allowed to diffuse from the disk by placing the plates with lids partially removed in a laminar flow hood for several hours and then incubating the plates, with lids on, at room temperature overnight. Alternately, if a laminar flow hood is unavailable, the plates can be incubated media-side up, lids closed, at room temperature overnight.

(4) The susceptibility plates may then be used immediately or stored in sealed plastic bags at 4 to 8 ºC for no more than 28 days. Protect all plates from light during storage.

(5) Test samples of each batch of plates for sterility by incubating at 35 ºC for 48 hours; discard these samples.

3.2.2.3 Medium with Liquid Drug

(1) Thaw a tube of the frozen stock of the drug and dilute with sterile distilled water to yield a working concentration (usually 200 to 10,000 µg/mL).

(2) To achieve the desired final concentration (see Table 2), add the appropriate volume of working solution as shown in Table 2 to 180 mL of sterile 7H10 agar tempered in a water bath at 50 to 56 °C. Tighten cap and mix by inversion of the tube.

(3) Add OADC (20 mL for a 200-mL total volume) and mix again.

(4) Dispense 5-mL amounts into labeled quadrants of a series of sterile plastic petri plates. One quadrant of each plate should be reserved for 7H10 agar medium without any added drug to serve as a growth control.

(5) Dispense the media onto the plates as quickly as possible after mixing the component parts to prevent partial solidification of the agar in the mixing container. Avoid production of bubbles. The agar in each quadrant should be 3 to 4 mm deep. Allow the agar to solidify at room temperature.

(6) Before use or storage, plates should be thoroughly dried by placing the plates with lids partially removed, in a laminar flow hood for several hours until the surface is dry. Avoid overdrying as this will alter the final drug concentration.

(7) After drying, use the plates immediately, or store them in sealed plastic bags at 4 to 8 °C for no more than 28 days. Protect all plates from light during storage.

(8) Test one or more plates from each batch of plates for sterility by incubating at 35 ºC for 48 hours; discard these samples.

3.2.3 Indirect Susceptibility Test

For the agar proportion method, susceptibility testing usually is performed using cultures already growing in a liquid medium or on the surface of a solid medium. The preparation of a standard inoculum is critical, because variations in the number of bacilli in the inoculum can alter the interpretation of the test.

3.2.3.1 Preparation of the Inoculum

3.2.3.1.1 Inoculum from Solid Media for the Agar Proportion Method

The following steps should be followed:

(1) The inoculum may be prepared by scraping freshly grown colonies (as early as adequate growth is attained but not more than four to five weeks old) from the surface of the agar medium, taking care to sample all parts of the growth. Care should also be taken not to scrape off any medium. Primary cultures, rather than subcultures, should be used whenever possible. Subcultures grown in broth may contain an insufficient number of slowly growing resistant tubercle bacilli in the culture, thus giving a "false-susceptible" result.

(2) Transfer the selected bacterial mass to a sterile 16- x 125-mm screw-cap tube containing 6 to 10 glass or plastic beads in 3 to 5 mL of tween-albumin liquid medium, such as Middlebrook 7H9.

(3) Emulsify the growth along the inside wall of the tube using a spatula or applicator stick. After tightly closing the cap, the contents of the tube are homogenized by vigorous agitation on a vortex mixer for one to two minutes, using sufficient speed to obtain only swirling, centrifugal mixing rather than churning, which may result in increased aerosol production.

(4) Allow the tube to stand for 30 minutes or longer in a test tube rack to allow larger clumps of organism to settle and to decrease the possibility of aerosol dispersion when the tube is opened.

(5) Withdraw the supernatant suspension and transfer it to another sterile glass tube. Adjust the absorbance by adding broth until the density is equivalent to that of a McFarland No. 1 turbidity standard. McFarland turbidity standards may be purchased commercially or prepared in-house using barium chloride and sulfuric acid (see Appendix B).

(6) Freshly grown primary cultures in broth, usually 7H9, may be used to prepare the inoculum. After mixing well, allow the suspension to settle for 30 minutes to reduce any aerosol at the top of the tube and allow larger clumps to settle. Then adjust the turbidity of the supernatant to be equivalent to the McFarland No. 1 standard by adding sterile medium.

3.2.3.2 Inoculation and Incubation of Media

3.2.3.2.1 7H10 Agar Plates

To inoculate the plates, perform the following steps:

(1) Prepare separate 10^{-2} and 10^{-4} dilutions of the standardized suspension in tween-albumin broth (such as 7H9), sterile saline, or sterile water.

(2) Using a sterile safety pipet, inoculate 0.1 mL of the 10^{-2} dilution onto the control quadrant and onto each of the drug-containing quadrants (this can be done by inoculating three drops at different points on each quadrant of the agar plate).

(3) Similarly, inoculate 0.1 mL of the 10^{-4} dilution onto the control quadrant and onto each of the drug-containing quadrants in a second series of drug-containing plates.

(4) If the culture to be tested is older than four to five weeks or scant growth is present, it may be necessary to use lower dilutions of the inoculum, such as 1:10 and 1:1000, or to subculture the organism first in broth. For broth subculture, a portion of the primary culture on solid medium is inoculated into 7H9 broth. The broth is incubated at 37 ± 1 ºC, with daily observation and mixing until the turbidity matches that of a No. 1 McFarland standard.

(5) Allow the inoculated plates to stand at room temperature (agar-side up with lids on) until the inoculum liquid has absorbed into the agar (i.e., until the spots are dry).

(6) Seal each plate in a CO_2 –permeable polyethylene bag.

(7) Incubate the plates agar-side down, at 37 ± 1 ºC in an atmosphere of 5 to 10% CO_2. Incubation in 5-10% CO_2 does not have a detrimental effect on the antimycobacterial drugs tested routinely in 7H10 agar medium. To prevent production of formaldehyde gas, plates should not be exposed to sunlight.

(8) Examine the plates carefully, using a dissecting microscope, each week for a period of no longer than three weeks. If there are at least 50 colonies (1+) on the control medium and the colonies are mature, resistance may be reported before three weeks. However, the interpretation "drug susceptible" should not be made until the third week to provide adequate time for growth.

Alternately, a modified indirect susceptibility method that requires less media can be considered. Organism dilutions of 10^{-2} and 10^{-4} are prepared as described above. One control quadrant without drug and all drug-containing quadrants are each inoculated with 0.1 mL of the 10^{-2} dilution. A second drug-free control is inoculated with 0.1 mL of the 10^{-4} dilution, which generally gives countable colonies (see Example 3 in Section 3.2.3.3.1).

3.2.3.3 Interpretation

The pathobiology of MTBC differs from that of many other bacteria. MTBC is an intracellular pathogen, and in this regard it is important to be aware that the intracellular drug concentration and activity may differ considerably from the corresponding values in serum and/or other extracellular fluids. Also, the infecting bacilli often are composed of differing mixtures of populations of actively growing, slowly growing, and latent organisms at different sites and inside walled-off tubercles; drug effectiveness may vary among these different populations. As a consequence of these aspects of MTBC infection, *in vitro* susceptibility testing of MTBC differs from that of aerobic and facultative bacteria in the following ways that directly or indirectly impact the reporting of results:

- Testing with any antimycobacterial agent is performed at one or two different concentrations.

- There is not uniform consensus regarding the clinical relevance of the results of testing at a higher concentration when two concentrations are used.

- "Critical" concentrations of certain drugs (the *in vitro* concentrations thought to be most relevant for predicting clinical responsiveness) were established many years ago, and for some drugs the values for these concentrations differ depending on the testing medium used.

- The reference agar proportion method employs a percentage calculation to determine resistance or susceptibility.

- The reference BACTEC 460TB method for PZA susceptibility testing employs a calculation procedure unique to that drug to determine resistance or susceptibility.

Many users of susceptibility reports may be confused or even misled if only the results of growth at the tested concentration(s) or an MIC is reported without some interpretive comment. Therefore, at a minimum, for every drug tested, reports should include the name of the drug tested, as well as a clinically helpful interpretive comment, such as "susceptible," "resistant," or "borderline," the last for PZA only. If a laboratory wishes to report the concentration at which each drug was tested, it should also specify the testing medium and/or testing method used, and/or specify the equivalent reference method concentrations. (If the equivalent reference method concentrations are given, then stating the actual concentrations tested and/or the testing method is optional.) Laboratories using the reference agar proportion method also have the option of reporting percent resistance, if they so choose. However, at this time there is no evidence to suggest that a lower percent resistance may indicate partial drug efficacy in the clinical management of the patient. To avoid confusion, whenever testing is performed at concentrations in addition to the "critical" concentrations (or their equivalents in methods other than the reference agar proportion method), the concentrations and method, or the equivalent reference method concentrations, should be specified.

In the case of an organism tested only against the low (critical) concentration of INH, to which it is resistant, the organism should be retested against the higher and critical concentrations of INH, and the following comment should be appended to the results: "This test result indicates the presence of at least low-level resistance to INH. Testing at a higher level of INH will be performed. A specialist in the treatment of tuberculosis should be consulted concerning the appropriate therapeutic regimen and dosages."

In the case of an organism tested against two concentrations of INH, to the lower concentration of which the organism is resistant and to the higher of which it is susceptible, the following comment should be appended to the results: "These test results indicate low-level resistance to INH. Some experts believe that patients infected with strains exhibiting this level of INH resistance may benefit from continuing therapy with INH. A specialist in the treatment of tuberculosis should be consulted concerning the appropriate therapeutic regimen and dosages."

In the case of an organism tested against two concentrations of INH, to both of which it is resistant, the following comment should be appended to the results: "These test results indicate high-level resistance to INH. A specialist in the treatment of tuberculosis should be consulted concerning the appropriate therapeutic regimen and dosages."

Some scenarios and examples of possible reports are shown in Appendix C.

3.2.3.3.1 Interpretation of Growth Observed on 7H10 Agar Plates

The amount of growth in each quadrant is recorded as follows:

>500 colonies (confluent growth)	4+
200-500 colonies (almost confluent growth)	3+
100-200 colonies	2+
50-100 colonies:	1+
<50 colonies:	record the actual number of colonies

At least one of the control quadrants of the set of two dilutions should have a minimum of 50 colonies; otherwise, the results are not valid, and the test must be repeated. If the plate with ≥ 50 colonies on the control is not the same as the dilution with ≥ 50 colonies for the drug-containing quadrants, one can use the number of countable colonies from the higher dilution plates, multiply this number by the dilution

difference between the two plates, and use this count as the denominator when calculating the percent resistance (see Example 2 in this section).

If the control quadrant has 3+ or 4+ growth and there is no growth in the drug-containing quadrant, the results can be reported as susceptible. In most cases, it will be possible to estimate the proportion of resistant colonies as greater or less than 1% of the control population. Most of the culture results will be obviously susceptible or resistant (see examples below), and only in rare instances should there be an ambiguous result.

Interpretation of the modified indirect susceptibility method follows guidelines for interpretation of the standard indirect method. See Example 3 for a sample calculation and interpretation.

The presence of microcolonies may represent true resistance, partial resistance, or may be a result of drug degradation followed by an overgrowth of susceptible organisms. One study reported that most strains that had microcolonies with EMB in the agar proportion method had EMB-susceptible results with BACTEC 460TB.[23] The significance of microcolonies is unknown. Since the frequency of microcolonies may vary from one laboratory to another, each laboratory should determine how to best report results. One approach is to always note the presence of microcolonies with a statement that their significance is unknown. If a laboratory opts not to report microcolonies associated with a specific drug (such as EMB) this decision should be based on its own experience with microcolonies (e.g., reproducibility on repeat testing) and consultation with its specialist in the treatment of TB.

The first reading at seven days is for the purpose of detecting the growth of contaminating bacteria or fungi, and for the detection of any rapidly growing mycobacteria. In the direct-susceptibility test, the growth of slowly growing mycobacteria may be evident within two weeks of incubation. Susceptibility test results should not be reported on readings made after this short incubation time, with the exception of a strain that is obviously resistant to the drugs, since drug-resistant tubercle bacilli can grow more slowly than susceptible strains. The optimal time for interpreting growth on plates is three weeks after inoculation. If the culture in the drug-free control has not grown at the three-week reading, the test should be repeated, preferably with a broth method. Simultaneously, the plates could be reincubated for three more weeks up to a total period of six weeks. The results of the test at the six-week reading, however, can be reported only for the agents to which the isolate appears to be susceptible, and if adequate growth exists in the drug-free quadrant. The reasons for this are not completely understood, but it is probable that the late-growing colonies escape drug action and begin to grow when the drug level in the medium degrades below the minimal inhibitory concentration.

The formula for determining percentage of resistance and some examples are:

$$\% \text{ Resistant} = \frac{\textit{number of colonies on drug-containing quadrant}}{\textit{number of colonies on control quadrant}} \bullet 100$$

Example 1. Sample Calculation and Interpretation

	Growth on:		
Antimycobacterial Agent/Concentration	**10^{-2}**	**10^{-4}**	**% Resistant**
Control	4+	100 colonies	—
INH (0.2 µg/mL)	2+	10 colonies	10
RIF (1.0 µg/mL)	0	0 colonies	0
EMB (5.0 µg/mL)	0	0 colonies	0

$$\% \text{ Resistant to INH} = \frac{\textit{10 colonies on drug-containing quadrant}}{\textit{100 colonies on control quadrant}} \bullet 100 = 10\%$$

Interpretation based on calculation, above: susceptible to RIF and EMB; 10% resistance to INH.

Example 2. Sample Calculation and Interpretation

	Growth on:		
Antimycobacterial Agent/Concentration	**10^{-2}**	**10^{-4}**	**% Resistant**
Control	4+	50 colonies	—
INH (0.2 µg/mL)	100 colonies	0 colonies	2
RIF (1.0 µg/mL)	0	0 colonies	0
EMB (5.0 µg/mL)	0	0 colonies	0

$$\% \text{ Resistant in INH} = \frac{\textit{100 colonies on drug-containing quadrant}}{\textit{50 colonies on control quadrant, multiplied by the dilution factor of 100, which is the difference between } 10^{-4} \textit{ and } 10^{-2}} \bullet 100 = 2\%$$

Interpretation based on calculation above: susceptible to EMB and RIF; 2% resistance to INH.

Example 3. Sample Calculation and Interpretation of the Modified Indirect Proportion Method[b]

	Growth on:		
Antimycobacterial Agent/Concentration	**10^{-2}**	**10^{-4}**	**% Resistant**
Control	4+	50 colonies	—
INH (0.2 µg/mL)	2+	NI*	>1
INH (1.0 µg/mL)	0	NI	0
RIF (1.0 µg/mL)	0	NI	0
EMB (5.0 µg/mL)	25 colonies	NI	<1

* NI – not inoculated (see Section 3.2.3.2)

Interpretation: Resistant to INH (0.2 µg/mL) (because colony counts [2+] are greater than the 10^{-4} control [50 colonies]); and susceptible to EMB (because colony counts [25 colonies] are less than the 10^{-4} control [50 colonies]), INH (1.0 µg/mL), and RIF.

3.2.4 Direct Susceptibility Test

3.2.4.1 Principle

The direct drug susceptibility test is a procedure based on inoculation of drug-containing media directly with a processed (concentrated after digestion and decontamination) sputum specimen that is smear-positive for acid-fast bacilli (AFB) to determine the proportion or percentage of resistant MTBC in the patient's bacterial population. The test should be performed only on smear-positive specimens, and only by the agar proportion method or by a commercial broth system that has been reviewed and cleared by FDA for direct susceptibility testing. Unless manufacturers obtain FDA clearance for these indications, individual laboratories testing direct specimens will need to conduct a study to establish, rather than just to verify, test performance characteristics to satisfy CLIA requirements.

[b] Hawkins JE. Drug susceptibility testing. *In*: Kubica GP, Wayne LG, eds. *The Mycobacteria: a Sourcebook*. New York, NY: Marcel Dekker, Inc.; 1984. Reprinted with permission from Marcel Dekker, Inc.

The advantages of the direct test are:

- Results can be reported within three weeks (from the time of specimen receipt in the laboratory) for a majority of smear-positive specimens.
- The proportion of resistant bacteria recovered better represents the patient's bacterial population.
- It is cost-efficient, because the materials are less expensive than those for rapid broth methods.

The disadvantages of the direct test are:

- The inability to accurately calibrate the inoculum, which may result in insufficient or excessive growth on drug-free control quadrants.
- Possible growth of normal respiratory flora that may survive digestion and decontamination, making results uninterpretable.
- The results of the test are valid only if the isolate is MTBC or *M. kansasii* (RIF only). Results for other species should not be reported.

The total rate of failure for the direct method can reach 10 to 15% or more, which results in retesting by one of the indirect methods.

3.2.4.2 Agar Plates

The direct susceptibility test is performed using 7H10 or 7H11 agar. The number of drug-containing plates to be inoculated will vary depending on whether only primary or both primary and secondary agents are to be tested.

3.2.4.3 Inoculation and Incubation

After the digestion and decontamination steps, and confirmation that the specimen is AFB smear-positive, the sputum specimen is inoculated onto drug-free (control) and drug-containing quadrants of the agar medium. The inoculum used is based on the results of the AFB smear, performed using a fluorochrome stain, as shown in Table 4. Each quadrant is inoculated with 0.1 mL of inoculum, except for specimens containing less than five AFB/field, in which case the inoculum is increased to 0.2 mL. After inoculation, the plates are treated as described in steps 5 to 8 in Section 3.2.3.2.1.

The plates are examined microscopically, using a dissecting microscope, without removing the plates from the polyethylene bags, at one, two, three, and six weeks of incubation. The results observed at one and two weeks of incubation are recorded onto a laboratory worksheet, but are not to be reported. The purpose of this examination is to evaluate for growth of contaminating normal respiratory flora and to determine (at two weeks) if a sufficient number of microcolonies are present on the drug-free medium. Contamination or insufficient growth suggests that the direct test will fail. In such cases, an indirect test (preferably a commercial rapid broth method with a short incubation time) can be initiated using primary growth from the initially inoculated media.

3.2.4.4 Reporting and Interpretation

Results are reported after three weeks of incubation, if the colonies on drug-free medium are mature, as described in Section 3.2.3.3.

Some isolates may not grow, or may not have sufficient growth for adequate interpretation after three weeks of incubation. If this occurs, the plates should be reincubated and examined at six weeks. "Susceptible" results can be reported at six weeks if no growth appears on the drug-containing segment. Apparent resistance at six weeks must not be reported; the test is considered invalid, since the growth after this prolonged period of incubation may be a result of partial degradation of the antimycobacterial agents.

3.3 Commercial Broth Systems with Shorter Incubation Time for Susceptibility Testing of MTBC

Users of this document should be aware that the standardized agar proportion method for the MTBC described here is not a rapid susceptibility testing method. To assure the earliest possible detection of resistant organisms, a commercial susceptibility test system with a shorter incubation should be used for testing isolates of MTBC obtained from any patient.[14] Any such commercial system utilized should have been previously demonstrated to produce results that correlate with those obtained with the standardized agar proportion method. The commercially available shorter-incubation systems that have been cleared for use by the U.S. FDA for use in the United States are listed in Appendix D. Laboratories should choose a broth-based system from a manufacturer that performs regular quality assurance testing to assure that medium and drug performance is consistent from one lot to the next and that medium and drug lots provide consistent results over longer periods of time (e.g., years) without drift. When making a decision between different shorter-incubation broth systems, laboratorians may ask the manufacturers to describe the measures they take to assure lot-to-lot consistency in drug susceptibility results, and weigh this information into their decision about which system to adopt. If any result obtained for an initial patient isolate with a commercial shorter-incubation system is questionable, then repeat testing using the standardized agar proportion method may be warranted. The standardized agar proportion method may be the only procedure that can be used for testing antimycobacterial agents for which no commercial susceptibility testing method has been demonstrated to produce results that correlate with those obtained by the standardized agar proportion method. Currently available commercial shorter-incubation systems are not FDA-cleared for testing slowly growing NTM; however, susceptibility test results for the slowly growing NTM are not so critical for either patient-treatment or epidemiological concerns. The susceptibility testing procedure described in Section 5 of this document for use with rapidly growing mycobacteria will yield results within several days of isolation of the organism.

3.4 PZA Susceptibility Testing

PZA is a first-line drug for the treatment of tuberculosis. Consideration should be given to testing all initial patient isolates of MTBC for susceptibility to this agent, but as the incidence of resistance to PZA remains very low, testing of isolates susceptible to other first-line drugs may not be necessary in areas where PZA resistance has not been documented. Agar-based methods such as the agar proportion method described elsewhere in this document have not proven to be satisfactory for PZA susceptibility testing, because many isolates fail to grow when the agar has been acidified to the degree necessary for PZA susceptibility testing. Susceptibility testing in the BACTEC 460TB system has proven to be satisfactory and is presently considered the reference method for PZA susceptibility testing.[24]

The procedures for PZA susceptibility testing using the BACTEC 460TB system are described in the manufacturer's instructions and should be followed scrupulously. The same considerations apply to the reporting of PZA results as for other antimycobacterial agents. Note, however, that unlike the case for any other antimycobacterial agent, a "borderline" category exists for the reporting of PZA results (see the manufacturer's instructions for use).

If an isolate is resistant only to PZA, consideration should be given to confirming or further defining its identification, (e.g., *Mycobacterium bovis* is resistant to PZA, whereas most isolates of *M. tuberculosis* (in the strict sense) are susceptible to PZA).

3.5 Quality Control (QC)

3.5.1 Purpose

The goal of a quality control program is to monitor the precision and accuracy (trueness) of the susceptibility test procedure, the performance of reagents used in the test, and the performance of those persons who carry out tests and read the results.

This goal is best realized through the use of reference strains selected for their genetic stability and for their usefulness in the particular method being controlled.

3.5.2 Selecting Reference Strains

Quality control of MTBC susceptibility testing should include an isolate that is completely susceptible to the antimicrobial agents being tested. The strain *M. tuberculosis* H37Rv (ATCC® 27294) (see Section 3.5.3) is well suited for use as a "pan-susceptible" control organism and its performance is well documented. Alternately, *M. tuberculosis* H37Ra, which is believed to be avirulent and therefore is less likely to cause laboratory-acquired infections in the event of an accident, could be used if a laboratory has documentation that this strain performs as expected. An advantage of using this strain is that its unique HPLC pattern is easily detectable, should evidence of the possibility of cross-contamination become an issue.

Some investigators suggest that laboratories also consider testing a resistant strain. In particular, those testing both the critical concentration and a higher concentration of INH should consider testing a strain (or isolate) of MTBC or a nontuberculous mycobacterium that consistently demonstrates resistance to the low concentration of INH but is susceptible to the higher concentration, if available. At present, however, it is not clear which strain (or isolate) would perform optimally for this purpose. Currently, a single strain or strains that are avirulent or have reduced virulence, pose minimal handling risks, and exhibit low levels of resistance to antimycobacterial drugs at or near the breakpoint have not been identified. Research to address this issue is needed. While single-drug-resistant strains are available, these strains are resistant to high concentrations (50 to 1,000 µg/mL) of drug and are not good candidates for QC purposes. Multiple-drug-resistant strains are available, but they pose an undesirable potential safety risk associated with frequent use and, therefore, are not suitable for use in most clinical laboratories. Use of a single MTBC strain that is resistant to more than two drugs is not recommended because of the serious, potentially life-threatening consequences of a laboratory-acquired infection.

3.5.3 Source of Reference Strains

Reference strains may be purchased from the American Type Culture Collection (ATCC®; P. O. Box 1549, Manassas, VA, 20108; www.atcc.org).

For those laboratories that voluntarily choose to adopt QC methods that include using drug-resistant strains, there are at least two possible sources for these isolates. In-house isolates with verified, reproducible low levels of resistance to one or two drugs may be maintained for use in testing. Another source is through participation in an external proficiency or performance testing program, such as the programs developed by the CDC and the College of American Pathologists (CAP).

3.5.4 Storing Reference Strains

Reference strains are to be stored in a way that minimizes the possibility of mutation in the organism resulting from repeated subculturing. Stock vials of strains can be prepared as suspensions (from solid or liquid cultures) in liquid medium (e.g., Dubos tween-albumin liquid medium), distributed in small

aliquots in multiple vials, and frozen preferably at –70 °C, although –20 °C is acceptable for short periods (e.g., 3 months).

To prepare inocula for QC purposes, one of the above vials is thawed, inoculated into several tubes of liquid medium, and incubated until turbidity is visible. The tubes are swirled for two to three minutes on a vortex-type mixer, and the contents are allowed to settle for at least 30 minutes. The supernatant fluid is removed, pooled, dispensed as 0.5- to 1-mL samples, and frozen at -70 °C. One of these samples is then thawed and multiple dilutions are plated in 0.1-mL amounts per quadrant to determine the dilution which yields 100 to 200 colonies. This dilution can be used for later routine QC (e.g., with each batch of agar proportion drug susceptibility tests). If viability decreases over time, the dilution can be adjusted to maintain the desired count. When vials near depletion, or the dilution must be adjusted more than tenfold to obtain the desired count, use another stock vial as inoculum to prepare a new batch of frozen samples.

3.5.5 Frequency of Testing

Lot numbers of all media components should be recorded. Each new lot of drug or media component, especially OADC and 7H10 powder, should be tested with the reference strains prior to use for patient testing. Also, reference strains should be used each time a new batch of test medium is prepared, and at least once a week, or with each run, both for QC purposes and to ensure QC strain stability during storage.

Three detailed assays that measure the comparative quality of media components are described in the literature.[25] They are as follows: a comparative resistance assay to monitor drug stability in solution and in agar; a disk-potency assay to monitor the potency of disks impregnated with antimycobacterial agents; and a standard concentrations assay to monitor changes in antibiograms caused by changes in the test medium.

Quality control testing using *M. tuberculosis* H37Rv (ATCC® 27294) should be performed once each week that patient isolates are tested. With regard to testing resistant strains, until an appropriate resistant quality control organism is identified, laboratories should consider participation in an external proficiency testing program (e.g., those provided by CAP and CDC) that periodically includes MTBC challenges with low-level resistance to INH, as well as challenges resistant to the other antituberculous drugs.

3.5.6 Mycobacterial Purity Checks

It is important to assure that each susceptibility test is performed on a pure culture of a single species of *Mycobacterium*. A variety of procedures may be employed for mycobacterial purity checks, depending on the details of the culture and susceptibility testing techniques employed by the laboratory. Some suggested purity check methods are as follows:

1. When the agar proportion method is used, careful attention should be paid to the colony morphology of the organism to ensure that it is consistent with that expected for the identified organism.

2. A day prior to setting up a susceptibility test on an organism growing in a liquid medium, inoculate a chocolate agar plate. If no growth is observed after overnight incubation, it is unlikely that any contaminating non-acid-fast organism is present.

3. When susceptibility testing is set up in a liquid medium for a slowly growing mycobacterium from a culture growing in a liquid medium, no additional purity check (to assure that only one mycobacterial species is present) need be done if the mycobacterium is susceptible to all the drugs tested (in the case of a member of the MTBC), or if the susceptibility pattern is that which would be expected for the species being tested (in the case of another species of slowly growing mycobacterium). If the organism is resistant to any of the drugs tested (in the case of a member of the MTBC), or if the

susceptibility pattern is different from that which would be expected for the species being tested (in the case of another species of slowly growing mycobacterium), then a subculture should be performed from at least one of the vials containing an antimycobacterial agent to which the organism is unexpectedly resistant, to assure that the culture is pure and that the colonial morphology is consistent with that to be expected for the identified organism. Such a subculture should be planted on a medium that will support the growth of slowly growing mycobacteria, and the medium should be incubated at 37 ± 1 ºC in 5 to 10% CO_2 until clearly visible, mature colonies appear. The subculture medium should then be carefully examined to be sure that only one colony type of mycobacterium is present. Also, gram and acid-fast stains can be done on smears prepared from one of the vials in which there is unexpected growth, to look for the possible presence of contaminating organisms.

4. A 7H10 agar plate can be inoculated at the time a susceptibility test is set up. If unexpected resistance is detected, the purity check plate can be examined under a microscope to determine if more than one colony type is present. Such a plate can also be incubated longer and examined when the colonies are more mature. This purity check can be combined with gram and/or acid-fast smears performed on broth cultures showing growth in the presence of drug described in (3) above.

3.6 Implementing New Methods

When laboratories implement a new test method, it is desirable to validate that the test system is performing appropriately. The first step in validation of a new method is to review the literature for publications of studies of the new method. In cases where the new test method is under consideration for replacing an existing test method, the new method should be as accurate as the method now in use. The easiest way to do this is to find a literature reference in which the new method, the present method, and the reference method are all tested in parallel with a set of susceptible and resistant cultures. Agreement between the new method and the reference method should be assessed for both susceptible and resistant isolates. Agreement is not expected to be 100%, but should be at least as good for the new method as for the method previously in use. It may not be possible to find a literature reference in which both the new method and the method currently in use are compared with the reference method. In that case, it may be useful to estimate the predictive values for test results in the laboratory based on sensitivity and specificity data in the available literature references. The frequency of false-positive (false-resistant) results for each drug with the new method can be calculated from the specificity reported in the literature reference. The specificity for each drug will give an estimate of the percentage of false-resistant results. The frequency of false-resistant results is compared with the prevalence of true resistance to the drug in the test population. Whether the predictive value of a resistant result will be satisfactory, or whether resistant results with some drugs will require repeat testing should be determined. Likewise, the frequency of false-negative (false-susceptible) results will be apparent from the sensitivity reported in the literature reference. False-susceptible results are considered a serious problem, because they can lead a physician to rely on a drug to which the organism is resistant. Adoption of a new method in the laboratory should be considered only if the frequency of false-susceptible results is very low (i.e., no greater than that of the previous method). If the frequency of false-susceptible results exceeds 5%, clinicians who use the results of drug susceptibility testing as an aid in formulating treatment regimens for tuberculosis patients should be notified of the limited accuracy of the method.

At times, considerations other than accuracy may prompt laboratorians to choose a new method over their existing method. Such considerations could include cost, personnel time, elimination of radioactive materials from the laboratory, etc. However, it is important to assess the relative accuracy of the new method, so that:

- test results by the new method which have reduced accuracy can be repeated and/or
- the clinicians who use the results of drug susceptibility testing for making patient-care decisions can be made aware of the diminished reliability of the new test results. When the new method is

adopted, notifying all of the laboratory's physician clients and the local tuberculosis control program of any changes expected in the accuracy of results is suggested.

It is conceivable that a new testing method could be developed which is more accurate than the method currently in use and even more accurate than the reference method. In this case, agreement of results with previously used methods might not be the best way to validate the new procedure. Correlation of test results with performance of the drugs *in vivo* (i.e., treatment success or failure) could be used to validate the new method in this circumstance.

Once a new method has been chosen, the validation protocol should include performing the current test method and a new method in parallel for a series of patient isolates. For testing of MTBC, the protocol should assess whether the new method can reliably detect resistance for each antimycobacterial agent that is tested. For some drugs, such as INH, the new method should be capable of detecting low-level resistant strains that are most commonly encountered. Validation of MIC methods for nontuberculous mycobacteria (NTM) should assess the precision of each drug result with known patient isolates and quality control strains exhibiting MICs within the range of drug concentrations tested. With a new method, the laboratory should further validate test results for several months by confirming results with selected isolates (e.g., drug-resistant MTBC) by another method or in another laboratory. For most laboratories, this would involve checking the agreement of test results when resistant isolates are sent to a referral laboratory for further testing.

4 Susceptibility Testing of Slowly Growing Nontuberculous Mycobacteria

4.1 Introduction

The procedures described in this section of the document are intended for susceptibility testing of certain slowly growing NTM, i.e., *Mycobacterium avium* complex, *Mycobacterium kansasii,* and *Mycobacterium marinum*. These species were selected, because there are sufficient data on which to base general recommendations; there is little information on the correlation of *in vitro* susceptibility testing results and clinical outcome for most other slowly growing NTM. For the most part, the recommendations concerning selection of antimicrobial agents reflect the opinions of the American Thoracic Society (ATS) concerning appropriate therapy.[26] With regard to testing methods, however, sufficient data to adequately address all issues do not exist. Areas where additional research is needed are indicated for each organism.

Several other slowly growing NTM may cause human disease. Susceptibility testing of such isolates occasionally is requested, but should be considered only for those believed to be clinically significant (e.g., isolates from blood, other sterile body fluids, or tissues or multiple isolates from sputum).

Currently, the ATS recommends the following criteria for determining the clinical significance of NTM from respiratory specimens:[26] three positive sputum/bronchial wash samples on three separate days with negative smears for AFB, or two positive cultures and one positive smear are usually sufficient to confirm clinical significance. Alternatively, if only one bronchial wash is available with positive culture or AFB smear ≥2+, this is sufficient to establish clinical significance. Single positive cultures which are AFB smear negative and/or contain low numbers of organisms are likely to be clinically insignificant.

For *Mycobacterium terrae/nonchromogenicum, Mycobacterium xenopi, Mycobacterium malmoense,* and *Mycobacterium simiae*, the primary and secondary drugs and concentrations suggested below for *M. kansasii* should be tested, and the same interpretive guidelines should be used. Unfortunately, too few isolates have been tested to recommend a specific method for testing these species. Similarly, for the fastidious mycobacteria *Mycobacterium haemophilum, Mycobacterium genavense, Mycobacterium ulcerans*, too little is currently known to recommend a standard method for susceptibility testing. *Mycobacterium gordonae* rarely is clinically significant; therefore, susceptibility testing is rarely required and should be discouraged.

4.2 Susceptibility Testing of *Mycobacterium avium* Complex (MAC)

4.2.1 Indications for Performing Susceptibility Tests

Currently, uniform agreement concerning the indications for susceptibility testing of isolates of MAC does not exist. However, investigators who have extensively studied MAC disease have recommended that isolates be tested in the following situations:[26,27]

- clinically significant isolates from patients on prior macrolide therapy;
- isolates from patients who develop bacteremia while on macrolide prophylaxis;
- isolates from patients who relapse while on macrolide therapy; and
- initial isolates from blood or tissue (patients with disseminated disease) or from clinically significant respiratory samples (e.g., sputum or bronchoalveolar lavage fluid) to establish baseline values. If baseline testing is not performed, saving the isolate for future testing (if necessary) is strongly recommended.

Susceptibility testing should be repeated after three months of treatment for patients with disseminated disease and after six months of treatment for patients with chronic pulmonary disease, if the patient shows no clinical improvement or clinical deterioration and is still culture positive. If baseline testing was not performed, both initial and recent isolates should be tested concurrently.

4.2.2 Antimicrobial Agents

The only antimicrobial agents for which a correlation between *in vitro* susceptibility tests for MAC and clinical response has been demonstrated in controlled clinical trials are the macrolides (azithromycin and clarithromycin). Therefore, susceptibility of MAC isolates should be evaluated for these drugs only.[26] Clarithromycin can serve as the class drug for the macrolides, and to be most cost-effective, it is the only drug that need be tested. Azithromycin may also be tested; however, it is problematic due to poor solubility at the high concentrations of drug that must be used. There have been no prospective controlled clinical trials evaluating *in vitro* susceptibility testing of MAC to other drugs, but a retrospective analysis in one study showed no correlation between EMB, RIF, and clofazimine susceptibility test results and clinical outcome.[28]

4.2.3 Susceptibility Test Method

Susceptibility testing should be performed using a broth-based method, either macrodilution or microdilution. However, for azithromycin, macrodilution is preferred, because solubility is less problematic when using larger volumes of broth. For either method, the inoculum should be prepared using transparent colonies, if they are present. With regard to macrodilution, there is some consensus in the United States that the radiometric method using the 12B medium is accurate and reliable.[29] For microtiter dilution testing, either 7H9 broth or Mueller Hinton broth supplemented with OADC or OAD may be used.[30] Microtiter trays are incubated at 37 ± 2 °C in ambient air and examined after seven days. If growth is poor, trays should be reincubated and read again at 10 or 14 days.

Currently, controversy concerning the optimal pH at which to test the macrolides exists amongst different investigators. The activity of the macrolides is affected by pH, i.e., they are more active *in vitro* in mildly alkaline conditions (pH 7.3 to 7.4) than under mildly acidic conditions (pH 6.8, which is the pH of the commercially available radiometric 12B medium).[31] For this reason, testing in a broth medium at pH 7.4 has been suggested.[32] At this time, testing susceptibility of MAC isolates to macrolides in broth at either

pH 6.8 or pH 7.4 is acceptable, providing that recommendations for interpretation of the breakpoints listed in Section 4.2.4 are followed. However, if testing is performed at pH 7.3 to 7.4 using the commercial radiometric method, laboratory personnel must modify the 12B medium and conduct adequate quality control (as suggested in Section 4.2.5). A method that has been used successfully to alkalinize the standard 12B medium is described in Appendix E.

Drug concentrations to test vary depending on the method used. For clarithromycin, the minimum range of concentrations (at doubling dilutions) is 1 to 64 μg/mL for the microdilution method; the optimum range is 0.25 to 256 μg/mL. For testing patient isolates by the radiometric macrodilution method, clarithromycin concentrations of 2, 4, 8, 16, 32, and 64 μg/mL are proposed. Alternatively, testing only 4, 16, and 64 μg/mL in 12B medium at pH 6.8 or 4, 8, and 32 μg/mL in 12B medium at pH 7.3 to 7.4 is more cost-effective and should provide adequate data for patient management. For testing patient isolates to azithromycin by the radiometric macrodilution method, concentrations of 16, 32, 64, 128, 256, and 512 μg/mL are optimal. Alternatively, testing only 32, 128, and 512 μg/mL (at pH 6.8) is more cost-effective and should provide adequate data for patient management.

For the radiometric macrodilution method, concentrations of both clarithromycin and azithromycin used for quality control are different from those used to test patient isolates. They are 1, 2, and 4 μg/mL for clarithromycin tested in 12B medium at pH 6.8 (0.5, 1, and 2 μg/mL at pH 7.3 to 7.4) and 8, 16, and 32 μg/mL for azithromycin (pH 6.8).

4.2.4 Reporting Results

MIC values and an interpretation should be reported as shown in Table 5.

The interpretive criteria are based, in part, on a monotherapy trial of disseminated disease in humans.[33] Wild-type MAC isolates are uniformly susceptible to macrolides, but macrolide resistance (as defined above) develops within a few months with monotherapy and may develop with combination therapy. Molecular analysis of MAC isolates that have developed resistance to macrolides *in vitro* has shown that over 95% of isolates have acquired a point mutation in the V-domain of the 23S rRNA gene.[34-36] Therefore, clinically significant resistance may be defined as a clarithromycin MIC of > 32 μg/mL at pH 6.8 or > 16 μg/mL at pH 7.3 to 7.4 or an azithromycin MIC of > 256 μg/mL (pH 6.8).

Untreated wild strains of MAC are unlikely to be intermediate or resistant to macrolides. Therefore, it is recommended that such results should not be reported until confirmed by repeat testing, and/or the identity of the isolate is confirmed. A confirmed intermediate result may suggest that the patient has a mixed population of MAC organisms; these patients should be followed for emerging resistance.

4.2.5 Quality Control

M. avium (ATCC® 700898) is recommended for quality control of broth dilution testing of MAC. The range of acceptable results is 1 to 4 μg/mL for clarithromycin at pH 6.8 (0.5 to 2 at pH 7.3 to 7.4) and 8 to 32 μg/mL for azithromycin.

Working control cultures of MAC should be subcultured weekly or each time susceptibility testing is performed (if done less often than weekly). For storage, these stock cultures should be maintained preferably at -70 °C, although -20 °C is acceptable for short periods (e.g., 3 months) in an appropriate medium (e.g., 7H9 or trypticase soy broth with 15% glycerol), although the working stock culture may be stored at room temperature for up to one month.

The overall performance of the susceptibility test system should be monitored by testing the appropriate reference strain weekly; or each time the test is performed if it is done less often than weekly.

When using 12B medium at pH 7.3 to 7.4, the pH of the medium must be tested each time a new stock of tripotassium phosphate (see Appendix E) is prepared. A minimum of three vials of standard 12B medium are injected with 0.3 mL of tripotassium phosphate solution. Vials are then incubated at 37 ± 2 °C overnight (in CO_2 or air). An aliquot of the broth is removed, and the pH is measured. The expected range is pH 7.3 to 7.4. If the pH is outside of this range for any of the three vials, a new solution must be prepared. Vials of 12B medium at pH 7.3 to 7.4 may be stored at room temperature or in the refrigerator for up to six months.

4.3 Susceptibility Testing of *Mycobacterium kansasii*

The drugs clinically active against *M. kansasii* that are used routinely for therapy include INH, RIF, and EMB. Rifabutin is used in place of RIF in HIV-infected patients being treated with protease inhibitors.[26] MICs to these drugs for untreated strains will fall within a narrow range, and routine susceptibility testing is generally not needed. Treatment failure can occur and is associated with rises in MICs (resistance) to RIF and occasionally to INH and/or EMB as well.[37] Such resistance, however, invariably includes resistance to RIF.[37] Thus, susceptibility testing is important in patients who failed therapy or had a poor response to initial therapy. The commercial radiometric system,[38,39] the modified proportion method in 7H10 agar,[37,39] and broth microdilution[37,40,41] have all been used for testing with the primary treatment drugs. The critical concentrations of RIF (1 µg/mL) and EMB (5 µg/mL) used for testing MTBC both inhibit wild strains of *M. kansasii*, with good correlation between Middlebrook 7H10 agar and broth methods.[37-41] However, the MICs for INH for wild strains of *M. kansasii* have ranged from 0.5 to 5.0 µg/mL,[40,42] so the standard MTBC critical concentration of 0.2 µg/mL in Middlebrook 7H10 agar invariably shows resistance, and the 1.0-µg/mL concentration gives variable results, even with multiple cultures of the same patient strain. For that reason, testing of INH for *M. kansasii* is not recommended with either of these concentrations.[26] Given that treatment failure is associated with RIF resistance and drug treatment histories are generally unavailable to the laboratory, susceptibility to the single drug RIF is the only one currently recommended for primary testing, a recommendation previously also made by the ATS.[26] Isolates susceptible to RIF are all susceptible to rifabutin, and special testing is not required for patients being treated with protease inhibitors. Susceptibility testing should be repeated if patients remain culture positive after three months of appropriate therapy.

For secondary drug testing (for isolates resistant to the 1-µg/mL concentration of RIF) a total of eight drugs could be tested. These drugs and proposed concentrations are given in Table 6. There is limited experience with these drug concentrations and any of the test media for isolates of *M. kansasii*[40,41, 43-45]

Incubation should be for 7 to14 days at 37 ± 2 ºC in CO_2 or ambient air for broth and solid media, and for the radiometric procedure manufacturer's recommendations for MTBC should be followed. If broth or other alternative methods are used, results should be comparable to the agar proportion method. When testing macrolides, incubation in CO_2 should be avoided.

4.3.1 Quality Control

M. kansasii ATCC® 12478, *M. marinum* ATCC® 927, and/or *Enterococcus faecalis* ATCC® 29212 may be used for quality control of susceptibility testing of clinical isolates of *M. kansasii* to rifampin. Expected results for the QC strains are: *M. kansasii* ATCC® 12478, ≤1 µg/mL; *M. marinum* ATCC® 927, ≤ 0.25 - 1 µg/mL; and *Enterococcus faecalis* ATCC® 29212, 0.5 – 4 µg/mL. The selected reference strain should be tested weekly or each time the test is performed, if less often than weekly.

4.4 Susceptibility Testing of *Mycobacterium marinum*

The major indications for susceptibility testing of nontuberculous mycobacteria are variability in susceptibility to clinically useful antimicrobial agents and/or significant risk of acquired mutational

resistance to one or more of these agents. In general, neither of these applies to isolates of *M. marinum*; therefore, **routine susceptibility testing of this species is not recommended.** Drugs that have been used successfully as single agents for therapy of clinical disease caused by *M. marinum* are RIF, doxycycline, minocycline, trimethoprim-sulfamethoxazole, and clarithromycin; the combination of RIF and EMB has also been used.[26] Isolates of *M. marinum* have a very narrow range of MICs for each of these agents, and because disease due to *M. marinum* is usually localized and the number of organisms is low (95% of tissue biopsies are AFB smear negative), single-drug therapy is widely used and acquired mutational resistance is rare.[26]

Susceptibility testing of *M. marinum* may be considered for patients who fail several months of therapy and remain culture positive. Methods have included testing of RIF (1 µg/mL) and EMB (5 µg/mL) with the agar proportion method using Middlebrook 7H10 agar recommended for MTBC. The radiometric system has been studied against a small number of isolates, and in one study results correlated well with results for Middlebrook for RIF, EMB, and doxycycline.[38] Agar disk elution[46] (see Appendix F) and broth microdilution[41] have also been used. Testing of doxycycline and minocycline has been problematic because of a trailing endpoint and marked instability in agar.[46] Although clinical isolates require isolation at 28 to 30 ºC, most isolates adapt well to 37 ± 2 ºC and can be tested at either temperature.[46] However, if growth is poor at the higher temperature, testing should be repeated at the lower temperature. The period of incubation should be seven days. A list of possible drugs to be tested for susceptibility is provided in Table 7. Incubation in CO_2 or ambient air is acceptable except when testing a macrolide, in which case CO_2 should be avoided.

4.5 Susceptibility Testing of Miscellaneous Slowly Growing Nontuberculous Mycobacteria

4.5.1 Nonfastidious Species

A number of other slowly growing nontuberculous mycobacteria exist that may cause human disease, and for which susceptibility testing results in agar and/or broth have been reported. These include isolates of *M. terrae/nonchromogenicum, M. xenopi*,[47] *M. malmoense,*[48] and *M. simiae*. Testing should include the same primary and secondary drugs used for *M. kansasii*, with the use of the same interpretive criteria. Too few isolates have been tested to recommend a specific method for these species.

4.5.2 Fastidious Species

A number of fastidious species of slowly growing nontuberculous mycobacteria exist that have been associated with clinical disease, and for which some susceptibility testing has been done. This includes *M. haemophilum* (requires ferric ammonium citrate or hemin to grow), *M. genavense* (requires incubation for more than six weeks using the radiometric system),[49] and *M. ulcerans* (requires incubation for four to six weeks).[50] Too little is known about these species to recommend a standard method for susceptibility testing.

4.5.3 Quality Control

Currently there is no one optimal reference strain for quality control of susceptibility testing of the slowly growing NTM for drugs other than rifampin. Therefore each laboratory should establish its own in-house validation for these organisms.

5 Antimycobacterial Susceptibility Testing for Rapidly Growing Mycobacteria

5.1 Introduction

The procedure described in this section is intended for susceptibility testing of the rapidly growing mycobacteria. The recommendations are based on data from studies that have included *Mycobacterium fortuitum* group (*M. fortuitum, M. peregrinum, M. fortuitum* third biovariant complex), *M. chelonae,* and *M. abscessus*; however, they should also apply to *M. mucogenicum, M. smegmatis* group (*M. smegmatis, M. goodii, M. wolinskyi*), and clinically significant, pigmented rapid growers. The method described is the standard broth dilution method (see the current edition of NCCLS document M7—*Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically* for details on performance of broth microdilution for rapidly growing aerobic bacteria).[26, 51-58]

Broth microdilution testing of pathogenic, rapidly growing mycobacteria requires skill acquired through experience with the test method and knowledge of the expected susceptibility patterns of different species. Therefore, for laboratories that encounter these organisms infrequently, referring those isolates for which susceptibility testing is indicated to an established reference laboratory is recommended. For laboratories that choose to do susceptibility testing in-house, test performance must be evaluated. Currently, no proficiency testing program regularly includes the rapidly growing mycobacteria, although the CDC performance evaluation for susceptibility testing of *M. tuberculosis* occasionally does. The best alternative at present is comparison of test results with those from an experienced reference laboratory. This should be done on initial validation and at regular intervals thereafter to demonstrate continued proficiency. Additionally, identification of isolates to the species level or, at a minimum, differentiation of *M. fortuitum* group from *M. chelonae-abscessus* group is recommended.

5.2 Indications for Performing Susceptibility Tests

Susceptibility testing is indicated for any rapidly growing mycobacteria that are considered clinically significant (e.g., isolates from blood, tissue, and skin and soft tissue lesions).[26] These organisms (especially *M. abscessus*) may cause pulmonary disease, but they also may be recovered as a contaminant or transient colonizer. Therefore, not all rapidly growing mycobacteria recovered from sputum are clinically significant. Factors that increase the likelihood that an isolate from sputum is a true pathogen are recovery from multiple specimens, recovery in large numbers, or recovery from a specimen that is smear positive for AFB. Isolates recovered in low numbers from only one of multiple sputum specimens are not likely to cause disease and therefore, do not warrant susceptibility testing. Clinical failure to eradicate rapidly growing mycobacteria from almost any site (except respiratory) after six months of appropriate antimicrobial therapy necessitates the need to confirm species identity and perform repeat susceptibility testing.

The MIC obtained using a dilution test may tell a physician the concentration of antimicrobial agent needed at the site of infection to inhibit the infecting organism. The MIC, however, does not represent an *absolute value.* The "true" MIC is somewhere between the lowest test concentration that inhibits the organism's growth (that is, the MIC reading) and the next-lower test concentration. If, for example, two-fold dilutions were used and the MIC was determined to be 16 μg/mL, the "true" MIC would be between 16 and 8 μg/mL. Even under the best of controlled conditions, a dilution test may not yield the same end point each time it is performed. Generally, the acceptable reproducibility of the test is within one twofold dilution of the actual end point. To avoid greater variability, the dilution test must be standardized and carefully controlled as described herein.

MICs have been determined using concentrations derived traditionally from serial twofold dilutions indexed to the base 1 (e.g., 1, 2, 4, 8, 16 μg/mL, etc.). Other dilution schemes have also been used, including use of as few as two widely separated or "breakpoint" concentrations. The results from these

alternative methods may be equally useful clinically. When there is inhibition of growth at the lowest concentration tested, the true MIC value cannot be accurately determined and should be reported as equal to or less than the lowest concentration tested.

Whenever MIC results are reported with the purpose of directing therapy, an interpretive category (i.e., "Susceptible," "Intermediate," or "Resistant") should accompany the MIC result.

5.3 Antimicrobial Agents and Concentrations

Agents that should be tested against the rapidly growing mycobacteria are: amikacin (1 to 128 µg/mL), cefoxitin (2 to 256 µg/mL), ciprofloxacin (0.125 to 16 µg/mL), clarithromycin (0.06 to 64 µg/mL), doxycycline (0.25 to 32 µg/mL), imipenem (1 to 64 µg/mL), sulfamethoxazole (1 to 64 µg/mL), and tobramycin (against *M. chelonae* only, 1 to 32 µg/mL). Trimethoprim-sulfamethoxazole may be substituted for sulfamethoxazole. Linezolid (2 to 64 µg/mL) may also be tested.[59,60]

Agents other than those listed above appear to have *in vitro* activity against rapidly growing mycobacteria. Such agents include cefmetazole (no longer available in the USA); vancomycin (for *M. fortuitum*); kanamycin; gentamicin; meropenem; amoxicillin-clavulanic acid; and the newer 8-methoxyfluoroquinolones, moxifloxacin and gatifloxacin. For most of these drugs insufficient laboratory and/or clinical data are available to make testing or interpretive recommendations at this time.

5.4 Preparation of the Inoculum

To standardize the inoculum density for a susceptibility test, a $BaSO_4$ turbidity standard equivalent to a 0.5 McFarland standard or its optical equivalent (e.g., a latex particle suspension that is commercially available) should be used (see Appendix B).

5.4.1 Inoculum Suspensions

Suspensions are prepared by sweeping the confluent portion of growth on an agar medium with a sterile cotton swab. Growth on the swab is transferred to 4.5 mL of sterile water containing glass beads (e.g., 7 to 10 3-mm beads) until the turbidity matches the density of a 0.5 McFarland standard (e.g., by visual examination or by using a nephelometer). The suspension could also be prepared directly from a broth culture. Suspensions are mixed vigorously on a vortex mixer for 15 to 20 seconds. If large clumps remain, they should be allowed to settle and the supernatant used for the inoculum suspension. If inoculating plates without the broth already added (i.e., freeze-dried), the final inoculum (with an organism density of approximately 5×10^5 CFU/mL) is prepared by transferring 50 µL of the suspension to 10 mL of cation-supplemented Mueller-Hinton broth, and tubes are inverted eight to ten times to mix the suspension thoroughly.

Alternatively, if inoculating prepared plates containing antimicrobials in 100 µL of broth, calculate the volume of standardized suspension to be added to 36 mL of water diluent to obtain a final organism concentration of 1×10^5 to 5×10^5 CFU/mL (1×10^4 to 5×10^4 CFU per well in 0.1 mL volume). The volume depends on the delivery system that is being used. For example, when using a disposable plastic multipronged inoculating device that delivers 0.01 mL per well, the inoculum is prepared as follows:

(1) Prepare a suspension equivalent to a 0.5 McFarland standard (1.5×10^8 CFU/mL) and mix by inverting the tube or using a vortex mixer.

(2) Add 0.5 mL of this suspension to 4.5 mL of water diluent (1:10 dilution) = 1.5×10^7 CFU/mL, and mix.

(3) Add 4 mL from step (2) to 36 mL of water diluent (1:10 dilution) = 1.5 x 10^6 CFU/mL.

(4) Prongs deliver 0.01 mL into 0.1 mL in well (1:10 dilution) = 1.5 x 10^5 CFU/mL or 1.5 x 10^4 per well.

5.5 Susceptibility Test Method

Final inoculum suspensions are mixed well (by inverting the tube several times or vortexing) and poured into plastic troughs. Transfer 100 µL to each well of the microtiter tray. Cover inoculated trays with an adhesive seal and incubate at 30 ± 2 °C in ambient air. Inoculate nutrient agar plate (e.g., 5% sheep blood, trypticase soy agar) with a loopful of the final inoculum to check for purity. Examine trays after 72 hours, and for *M. fortuitum* group, this should be the only reading. If growth (appearing as turbidity or a deposit of cells at the bottom of the well) in the growth control well is sufficient (i.e., at least 2+, based on the scale illustrated in Figure 1) record the MIC. Otherwise, reincubate the tray and read daily thereafter (for up to five days total) until growth is sufficient. For all but one of the drugs recommended in Section 5.3, the MIC is the lowest concentration of drug that inhibits visible growth. The exception is sulfamethoxazole, for which the end point is the well with approximately 80% inhibition of growth compared to the growth in the control well with no drug (e.g., well 1B if 1E is the growth control as shown in Figure 1).

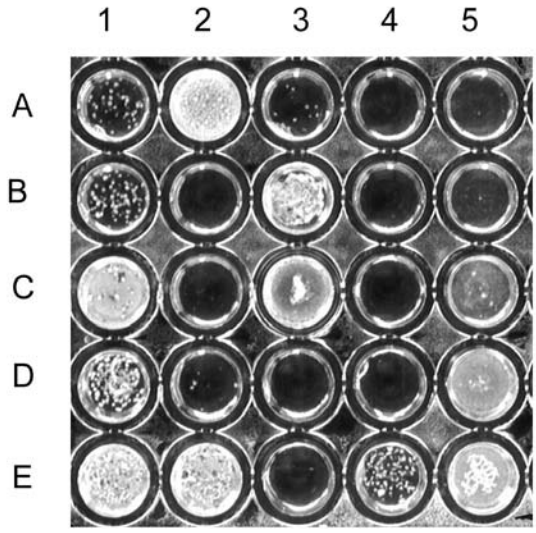

Figure 1. Guidelines for Interpretation of Broth Microdilution MIC Endpoints When Testing Rapidly Growing Mycobacteria. The ± sign denotes 1-5 countable colonies/flakes, and or slight but definite turbidity. Care must be taken to read known negative wells as a slight precipitate related to the inoculum may be seen.

1E Control growth (4+)
3A ± Few countable colonies; no turbidity (also well 5B, 2D are ±)
5C ± Few countable colonies; hazy turbidity (also well 3E is ±)
5D 1+ Definite haze or turbidity
5E 2+ Definite turbidity and "clumpy growth"
1B 2+ Moderate countable colonies; slight turbidity
1D 3+ Heavy colonial growth
4E 3+ Heavy growth; turbidity
1C 4+ Heavy confluent growth comparable to control (also wells 2A and 2E are 4+)

Wells 4A, 5A, 2B, 4B, 4C, 3D, 4D are all negative.

5.6 Reporting of MIC Results

MIC values should be reported with two exceptions: (1) Imipenem results should not be reported for *M. chelonae* and *M. abscessus* because of problems with reproducibility and interpretation. When testing isolates of *M. chelonae* and *M. abscessus*, incubation for four to five days is sometimes required, a need not evident with other groups or species or rapidly growing mycobacteria. Given the known temporal instability of imipenem, the need for more prolonged incubation when testing *M. chelonae* and *M.*

abscessus, and the usual MICs (i.e., 8 to 16 μg/mL) for these species being at the resistance breakpoint, imipenem MICs for these species tend not to be reproducible and to result in major category changes of susceptibility; therefore, results should not be reported for these two species.[51] In contrast, susceptibility results should be reported for other rapidly growing mycobacteria, including isolates of the *M. fortuitum* group, *M. mucogenicum,* and the *M. smegmatis* group. All isolates of these groups tested to date have been susceptible to imipenem *in vitro*,[61,62] and have not exhibited problems with interpretation and especially major category changes of susceptibility. (2) Tobramycin results should be reported only for *M. chelonae,* because therapeutically, this drug is superior to amikacin only for *M. chelonae* infections.[62] Because tobramycin is the aminoglycoside of choice for isolates of *M. chelonae*, amikacin results should be reported only if the isolate is resistant to tobramycin. Breakpoints for the rapidly growing mycobacteria are listed in Table 8.

The MIC values determined as described in this document may be reported directly to clinicians for patient care purposes. However, it is essential for an understanding of the data by all clinicians that an interpretive category result (i.e., "susceptible," "intermediate," "resistant" [defined in Section 2]) also be provided routinely. For most agents, these categories were developed by determining MICs of a large number of isolates, including those with known mechanisms of resistance relevant to the particular class of drug. Second, the MICs were analyzed in relation to the pharmacokinetics of the drug from normal dosing regimens. Third, whenever possible, the *in vitro* interpretive criteria were analyzed in relation to studies of clinical efficacy in the treatment of specific pathogens.

5.7 Quality Control Procedures

5.7.1 Purpose

The goals of a quality control program are to assist in monitoring the following:

- the precision (repeatability) and accuracy (trueness) of the susceptibility test procedure;
- the performance of reagents used in the test; and
- the performance of persons who carry out the tests and read the results.

The goals are best accomplished by, but not limited to, the testing of quality control strains with known susceptibility to the antimicrobial agents to be tested.

5.7.2 Quality Control Responsibilities

Modern laboratories rely heavily on pharmaceutical and diagnostic product manufacturers for provision of reagents, media, or test systems for the performance of antimicrobial susceptibility tests. Although this section is intended to apply to only the standard reference methods, it may be applicable to certain commercially available test systems that are based primarily, or in part, on these methods.

A logical division of responsibility and accountability may be described as follows:

(1) Manufacturers (in-house or commercial products):

- antimicrobial stability;
- antimicrobial labeling;
- potency of antimicrobial stock solutions;

- compliance with U.S. FDA quality system regulations;
- integrity of product; and
- accountability and traceability to consignee.

(2) Laboratory (user):

- storage under the environmental conditions recommended by the manufacturer (to prevent drug deterioration);
- proficiency of personnel performing tests; and
- adherence to the established procedure, e.g., inoculum preparation, incubation conditions, interpretation of end points.

Manufacturers should design and recommend a quality control program that allows users to evaluate those variables (e.g., inoculum density, storage/shipping conditions) that most likely could cause user performance problems and to determine that the test is performing correctly when used according to established protocols.

5.7.3 Reference Strains for Quality Control

Ideal reference strains for quality control of dilution tests have MICs that fall near the middle of the concentration range tested for all antimicrobial agents; e.g., an ideal control strain would be inhibited at the fourth dilution of a seven-dilution series, but strains with MICs at either the third or fifth dilution would also be acceptable. In certain instances with newer, more potent antimicrobial agents, it may be necessary to test additional quality control strains not normally tested in order to provide on-scale values.

Mycobacterium peregrinum ATCC® 700686 (tentative limits in Table 9), incubated for three days at 30 ± 2 °C, is recommended for controlling dilution testing of rapidly growing mycobacteria. Alternatively, until additional data are available, *Staphylococcus aureus* ATCC® 29213 may be used for quality control.

5.7.4 Storing Quality Control Strains

(1) Working control cultures of rapidly growing mycobacteria should be subcultured weekly (or with each test run if done less often than weekly) from frozen stocks. For storage, these stock cultures should be stored at -20 °C or below in an appropriate medium (e.g., tryptic soy broth with 15% glycerol), which should minimize the risk of altering their antimicrobial susceptibility.

(2) Before testing, the strains should be subcultured onto appropriate agar plates to obtain isolated colonies.

(3) Colonies are grown or suspended for testing according to the recommended inoculum-preparation procedures.

(4) A quality control culture may be used to monitor precision and accuracy (trueness) of dilution tests as long as there is no significant change in the MICs that cannot be attributed to faulty methodology. If an unexplained result suggests a change in the control organism's inherent susceptibility, a fresh culture of the frozen stock control strain should be obtained.

5.7.5 Batch or Lot Quality Control

(1) Each new batch or lot of microdilution trays should be tested with the appropriate reference strains to determine if MICs obtained with the batch fall within the expected range (see Table 9); if they do not, the batch must be rejected.

(2) At least one uninoculated microdilution tray from each batch should be incubated overnight to verify sterility of the medium.

(3) Records should be kept of the lot numbers of all materials and reagents used in performing susceptibility tests.

5.7.6 Quality Control Ranges

As above, please refer to Table 9. Results for drugs for which the QC result is not in range should not be reported.

5.7.7 Frequency of Testing

The overall performance of the susceptibility test system should be monitored by testing appropriate reference strains weekly or each day the test is performed (if testing is done less often than weekly).

5.7.8 Other Control Procedures

Each microdilution broth tray should include a growth control of basal medium without antimicrobial agent to assess viability of the test organisms. The growth control also serves as a turbidity control for reading end points.

5.7.9 Purity Control

For broth dilutions where mixed cultures are possible and likely to go unrecognized, testing for purity is suggested. Following inoculation of broth dilution tests, a sample from each inoculum is streaked on a suitable agar plate (e.g., chocolate agar) and incubated overnight to detect mixed cultures and with incubation continued for up to three days to provide freshly isolated colonies in case retesting proves necessary.

5.7.10 End Point Interpretation Control

End point interpretation is monitored periodically to minimize variation in the interpretation of MIC end points among observers. All laboratory personnel who perform these tests should independently read a selected set of dilution tests. The results are recorded and compared to the results obtained by an experienced reader. All readers should agree within ±1 concentration of one another.[63]

5.7.11 Quality Assurance Measures

Multiple test parameters are monitored by following the quality assurance guidelines described in this standard. Acceptable results derived from testing quality control strains do not guarantee accurate (true) results with all patient isolates. When atypical or inconsistent results are encountered with patient isolates, repeat testing and/or repeat identification procedures should be performed in an effort to ensure accurate (true) results. Each laboratory should develop its own policies regarding verification of atypical antimicrobial susceptibility testing results.

6 Susceptibility Testing of Aerobic Actinomycetes[64-71]

6.1 Method

The recommended method for susceptibility testing of the aerobic actinomycetes (*Nocardia* species, *Actinomadura* species, *Rhodococcus* species, *Gordona* species, *Tsukamurella* species, *Streptomyces* species, etc.) is broth microdilution (see the current edition of NCCLS document M7—*Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically* for details on performance of broth microdilution for rapidly growing aerobic bacteria). Inoculum preparation is identical to that described for the rapidly growing mycobacteria (refer to Section 5) except that suspensions may also be prepared from a blood agar plate that has been incubated at 37 ± 2 ºC in ambient air, overnight, or until growth is sufficient for testing. Incubation is at 37 ± 2 ºC in ambient air for three days, although some species (e.g., *Nocardia nova*, *Actinomadura* spp., etc.) may require incubation for up to five days.

6.2 Antimicrobial Agents

Drugs that are recommended for primary testing are: amikacin, amoxicillin-clavulanic acid, ceftriaxone, ciprofloxacin, clarithromycin, imipenem, linezolid, minocycline, sulfamethoxazole or trimethoprim-sulfamethoxazole, and tobramycin. Secondary drugs that may be tested include: cefepime, cefotaxime, doxycycline, gatifloxacin or moxifloxacin, and gentamicin.

6.3 Reporting of Results

Both an MIC value and an interpretation (i.e., "susceptible," "intermediate," "resistant") should be reported for each drug tested. Breakpoints for the aerobic actinomycetes are listed in Table 10.

6.4 Quality Control

The overall performance of the susceptibility test system should be monitored by testing appropriate reference strains weekly or each day the test is performed (if testing is done less often than weekly). The recommended reference strains for quality control are *Staphylococcus aureus* ATCC® 29213, and (for amoxicillin-clavulanic acid only) *Escherichia coli* ATCC® 35218. Acceptable ranges for these strains are those listed in the most current edition of NCCLS document M100—*Performance Standards for Antimicrobial Susceptibility Testing.*

Table 1. Antituberculous Drugs and Their Recommended Concentrations in Middlebrook 7H10 and 7H11 Agar Medium[18, 72]

	Concentrations (µg/mL)	
Primary Drugs	**7H10 agar**	**7H11 agar**
Isoniazid	0.2 1.0	0.2 1.0
Rifampin[*]	1.0	1.0
Ethambutol hydrochloride	5.0	7.5[†]
Pyrazinamide[‡]	NR	NR
Secondary Drugs[§]		
Capreomycin	10.0	10.0
Ethionamide	5.0	10.0
Ethambutol hydrochloride[¶]	10.0	10.0
Kanamycin[#]	5.0	6.0
Ofloxacin[**]	2.0	2.0
p-Aminosalicylic acid	2.0	8.0
Rifabutin[††]	0.5	0.5
Streptomycin	2.0 10.0	2.0 10.0

[*] Rifampin is the class representative for rifapentine.

[†] Data supporting equivalency with 7H10 (5.0 µg/mL) are limited.

[‡] NR=not recommended. For pyrazinamide testing, the manufacturer's directions for the BACTEC 460TB procedure should be followed (see Section 3.2.1.3).

[§] All secondary drugs should be tested on isolates of *M. tuberculosis* that are resistant to RIF or resistant to any two primary drugs. Testing cycloserine, which is an option therapeutically, is not recommended due to technical problems with the test.[72]

[¶] Ethambutol should be tested at this higher concentration as a secondary drug.

[#] Kanamycin is the class representative for amikacin.

[**] Ofloxacin is the class representative for fluoroquinolones.

[††] Some investigators have included a higher concentration, usually 1.0 to 2.0 µg/mL. The clinical significance of these concentrations, especially in the setting of RIF resistance, is unknown.

Table 2. Stock, Working, and Final Concentrations of Antituberculous Drug Solutions for Middlebrook 7H10 Agar Medium

Antimicrobial Agent	Potency* (µg/mL)	Solvent	Stock† Concentration (µg/mL)	Working Concentration (µg/mL) from Stock Concentration for 7H10 Agar	Volume of Working Concentration (mL) to Add to 200 mL 7H10 Agar	Final Concentration of Drug in 7H10 Agar
Capreomycin	Varies with lot	SDW	10,000	1,000	2.0	10.0
Ethambutol Hydrochloride	1,000	SDW	10,000	1,000	1.0 2.0	5.0 10.0
Ethionamide	1,000	DMSO	10,000	1,000	1.0	5.0
Isoniazid	1,000	SDW	10,000	200	0.2 1.0	0.2 1.0
Kanamycin	Varies with lot	SDW	10,000	1,000	1.0	5.0
Ofloxacin	1,000	SDW	10,000	200	2.0	2.0
Rifabutin	Varies with lot	Methanol	10,000	100	1.0	0.5
Rifampin	1,000	DMSO‡	10,000‡	1,000	0.2	1.0
Streptomycin sulfate	Varies with lot	SDW	10,000	1,000	0.4 2.0	2.0 10.0
p-Amino-salicylic acid	1,000	SDW	10,000	1,000	0.4	2.0

Please see next page for footnotes corresponding to Table 2.

Table 2. (Continued)

[*] Calculate the weight based on potency if less than 100% (described in Section 3.2.1.2).

[†] 100 mg of active drug (depending on the potency) dissolved in 10 mL of sterile distilled water will yield a stock solution of 10,000 µg/mL. Sterilize stock solutions by filtration through 0.22-µm pore membrane, dispense into sterile vials, and store up to 12 months at -70 ºC. Thaw to room temperature and use without delay, discard excess, and never refreeze. Lower concentration stock solutions and higher storage temperatures have also demonstrated satisfactory stability for 12 months (i.e., capreomycin 1,000 µg/mL at –20 ºC, ethambutol HCl 5,000 µg/mL at 3 to 7 ºC, isoniazid 200 µg/mL at 3 to 7 ºC, kanamycin 500 µg/mL at –20 ºC, streptomycin 2,000 µg/mL at 3 to 7 ºC, and PAS 2,000 µg/mL at 3 to 7 ºC). (Reference: Griffith ME, Bodily HW. Stability of antimycobacterial drugs in susceptibility testing. *Antimicrob Agents Chemother.* 1992;36:2398-2402).

[‡] An alternative solvent for RIF is N,N-dimethylformamide at 50,000 µg/mL, followed by dilution to working concentrations in tepid SDW; however, these RIF preparations have not been successfully stored.

SDW, Sterile distilled water.
DMSO, Dimethyl sulfoxide.

Table 3. Agar Proportion Method Using 7H10 Agar and Drug-Containing Disks for Susceptibility Testing of *Mycobacterium tuberculosis*

Drug	Amount (μg) per Disk	Final Drug Concentration (μg/mL)
Control	—	0
Isoniazid	1	0.2
Isoniazid	5	1.0
Ethambutol Hydrochloride	25	5.0
Rifampin	5	1.0

Table 4. Dilution of Sputum Concentrate for Inoculation of the Susceptibility Test Medium for the Direct Susceptibility Test[*]

No. of AFB/field (at 200x to 400x magnification) observed using:

Fluorochrome Stain	Dilutions
< 25[†]	undiluted
25-50	undiluted,1:10
50-250	undiluted, 1:100
> 250	1:100, 1:1000

[*] Inoculation of two sets of plates is recommended, if sufficient inoculation material exists.
[†] Increase inoculum to 0.2 mL for specimens with less than 5 AFB seen per field at 200 to 400x magnification.

Table 5. Macrolide Interpretative Criteria for *Mycobacterium avium* Complex

Drug	Method/pH	MIC (μL/mL) for Category		
		Susceptible	Intermediate*	Resistant
Clarithromycin	BACTEC 460 TB or broth microdilution (pH 6.8)	≤ 16	32	≥ 64
	BACTEC 460 TB (pH 7.3 – 7.4)	≤ 4	8-16	≥ 32
	Broth Microdilution† (pH 7.3 – 7.4)	≤ 8	16	≥ 32
Azithromycin	BACTEC 460 TB (pH 6.8)	≤ 128	256	≥ 512

***** For an isolate with an "Intermediate" level of susceptibility to one of the macrolides, a comment should be added to the report of the susceptibility result, indicating that the isolate should be monitored carefully for possible development of macrolide resistance. For example, if an isolate has a clarithromycin MIC of 8 μg/mL as determined by BACTEC 460TB at pH 7.3 to 7.4, the report should read essentially as follows:

Clarithromycin MIC by the BACTEC 460TB method at pH 7.3 to 7.4 is 8 μg/mL (Intermediate). An Intermediate macrolide susceptibility test result may indicate emerging resistance. The patient should be monitored carefully, and subsequent MAC isolates should be tested for possible development of macrolide resistance.

† Cation adjusted Mueller-Hinton broth (pH 7.3 –7.4) or Middlebrook 7H9 broth (pH corrected to 7.3 – 7.4 with 1M potassium hydroxide).

Table 6. Proposed Antimycobacterial Agents, MIC Values Indicating Resistance, and Media for Testing of *M. kansasii* as well as Selected References Used to Evaluate the Test Concentrations

Antimycobacterial Agent	MIC Indicating Resistance (µg/mL)	References Categorized by Test Method		
		Agar Proportion*	BACTEC 460TB	Microdilution (7H9, MHB + 5% OAD)†
Primary Agents				
Rifampin‡	1	42, 43, 44, 48 41, 45, 46§		41,45
	2		43 42	
Secondary Agents				
Rifabutin	2	41, 48		41, 45
Ethambutol Hydrochloride	5	41, 42, 44, 45, 46§	42	41, 45
Isoniazid¶	5	41, 42, 44, 45, 46§	42	41, 45
Streptomycin¶	10	41, 42, 44, 45, 46§	42	41, 45
Clarithromycin¶,#	16	47	47,49	41
Amikacin¶	32			41, 45
Ciprofloxacin¶,**	2			41
Trimethoprim - sulfamethoxazole Or Sulfamethoxazole¶	2/38			41, 45
	32			41, 45

* 7H10 agar was used in the references listed; data regarding 7H11 agar are insufficient for comment. Agar disk elution (see Appendix F) can also be used.

† MHB + OAD is cation-supplemented Mueller-Hinton Broth supplemented with 5% oleic acid, albumin, and dextrose.

‡ For patients who are taking protease inhibitors, isolates susceptible to rifampin can be assumed to be susceptible to rifabutin. The concentration of 1 µg/mL relates to agar proportion and microdilution methods; the concentration of 2 µg/mL relates to the BACTEC 460TB method.

§ Presumed to have been performed in agar.

¶ Limited information is available on testing of these drug concentrations and optimal testing conditions.

Class representative for "newer" macrolides (clarithromycin, azithromycin, roxithromycin).

** Ciprofloxacin may be used as a class representative for the older fluoroquinolones: ciprofloxacin, ofloxacin, and levofloxacin. However, ciprofloxacin is not as active *in vitro* as the new 8-methoxyfluoroquinolones (i.e., gatifloxacin and moxifloxacin) and cannot be used as the class representative for these agents.

Table 7. Proposed Antimycobacterial Agents, MIC Values Indicating Resistance, and Media for Susceptibility Testing of *Mycobacterium marinum* (Routine testing is not recommended.)

Antimycobacterial Agent	MIC Indicating Resistance (μg/mL)	Method[*]
Rifampin	1	Agar proportion; agar disk elution; broth microdilution (cation-supplemented Mueller-Hinton Broth with 5% OAD)
Ethambutol hydrochloride	5	As above, for rifampin
Doxycycline/Minocycline	6 4	Agar disk elution Broth microdilution
Clarithromycin[†]	16	Broth microdilution
Trimethoprim - sulfamethoxazole or Sulfamethoxazole	2/38 20 32	Broth microdilution Agar disk elution Broth microdilution
Amikacin	12 32	Agar disk elution Broth microdilution

[*] Susceptibility reports by any method are limited for these agents, and few comparative studies have been done. Agar disk elution is described in Appendix F.

[†] Class representative for macrolides (clarithromycin, azithromycin, roxithromycin).

Table 8. Broth Microdilution Interpretive Criteria for Rapidly Growing Mycobacteria

Antimicrobial Agent	MIC (µg/mL) for category: Susceptible	Intermediate	Resistant
Amikacin[*]	≤ 16	32	≥ 64
Cefoxitin	≤ 16	32-64	≥ 128
Ciprofloxacin[†]	≤ 1	2	≥ 4
Clarithromycin[‡]	≤ 2	4	≥ 8
Doxycycline	≤ 1	2-8	≥ 16
Imipenem[§]	≤ 4	8	≥ 16
Linezolid[¶]	≤ 8	16	≥ 32
Sulfamethoxazole[#]	≤ 32	-	≥ 64
Tobramycin[**]	≤ 4	8	≥ 16

NOTE: These breakpoints, with the exception of cefoxitin, doxycycline, and linezolid, are derived from aerobic dilution interpretive criteria found in the most current edition of NCCLS document M100—*Performance Standards for Antimicrobial Susceptibility Testing.* Cefoxitin interpretive criteria are derived from data found in reference 47; proposed linezolid interpretive criteria are derived from data found in references 56 and 57.

[*] Isolates of *M. abscessus* with an MIC of ≥64 µg/mL should be retested. If the repeat result is ≥64 µg/mL, the MIC should be reported with the comment: 1) The MIC is greater than expected for this species; and 2) if the drug is being considered for therapy, the laboratory should be notified so the isolate can be sent to a reference laboratory for confirmation of resistance.

[†] Ciprofloxacin may be used as a class representative for the older fluoroquinolones: ciprofloxacin, ofloxacin, and levofloxacin. However, ciprofloxacin is not as active *in vitro* as the new 8-methoxyfluoroquinolones (i.e., gatifloxacin and moxifloxacin) and cannot be used as the class representative for these agents. See Section 5.3 and Summary of Comments question and response number 23.

[‡] Isolates of *M. fortuitum* group with a trailing end point should be considered resistant. Results for *M. chelonae* and *M. abscessus* should be read at three days (no more than four days). Clarithromycin is the class representative for the newer macrolides (i.e., azithromycin, roxithromycin).

[§] Report for *M. fortuitum* group, *M. smegmatis* group, and *M. mucogenicum*. If the MIC is >8 µg/mL, the test should be repeated with incubation period of no more than three days. If the repeat result is >8 µg/mL, the MIC should be reported with the comment: 1) The MIC is greater than expected for this species; and 2) if the drug is being considered for therapy, the laboratory should be notified so the isolate can be sent to a reference laboratory for confirmation of resistance. MICs to imipenem should not be reported for *M. chelonae* or *M. abscessus.*

[¶] Proposed breakpoints[59]

[#] MIC is indicated by 80% inhibition of growth.

[**] Report for *M. chelonae* only. If the MIC is >4 µg/mL, the test should be repeated. If the repeat result is >4 µg/mL, the MIC should be reported with the comment: 1) The MIC is greater than expected for this species; and 2) if the drug is being considered for therapy, the laboratory should be notified so the isolate can be sent to a reference laboratory for confirmation of resistance.

Table 9. Tentative Quality Control Ranges of Minimal Inhibitory Concentrations (MICs) (µg/mL) for *Mycobacterium peregrinum* ATCC® 700686 and *Staphylococcus aureus* ATCC® 29213 (when testing rapidly growing mycobacteria)[*]

Antimicrobial Agent	MIC range (µg/mL) for *Mycobacterium peregrinum* ATCC® 700686 (preferred organism)	MIC range (µg/mL) for *S. aureus* ATCC® 29213 (alternate organism)
Amikacin	≤ 1-4	1-4
Cefoxitin	16-32	1-4
Ciprofloxacin	≤ 0.12-0.5	0.12-0.5
Clarithromycin	≤ 0.06-0.5	0.12-0.5
Doxycycline	0.12-0.5	0.12-0.5
Imipenem	2-16	NA
Linezolid	≤ 2-4	1-4
Sulfamethoxazole	≤ 1-4	32-128
Tobramycin	4-8	0.12-1

NA = not available

[*] As an alternative, until additional data are available, other strains and QC ranges that may be used for quality control can be found in Table 3 (*S. aureus*) in the most current edition of NCCLS document M100—*Performance Standards for Antimicrobial Susceptibility Testing.*

Table 10. Broth Microdilution Breakpoints for *Nocardia* and Other Aerobic Actinomycetes

Antimicrobial Agent	MIC (µg/mL) for category		
	Susceptible	Intermediate	Resistant
Primary			
Amikacin*	≤ 8	-	≥ 16
Amoxicillin-clavulanic acid	≤ 8/4	16/8	≥ 32/16
Ceftriaxone	≤ 8	16-32	≥ 64
Ciprofloxacin†	≤ 1	2	≥ 4
Clarithromycin‡	≤ 2	4	≥ 8
Imipenem	≤ 4	8	≥ 16
Linezolid§	≤ 8	-	-
Minocycline*	≤ 1	2-4	≥ 8
Sulfamethoxazole* or Trimethoprim-sulfamethoxazole (TMP-SMX)	≤ 32 ≤ 2/38	- -	≥ 64 ≥ 4/76
Tobramycin	≤ 4	8	≥ 16
Secondary			
Cefepime	≤ 8	16	≥ 32
Cefotaxime	≤ 8	16-32	≥ 64
Doxycycline	≤ 1	2-4	≥ 8
Gentamicin	≤ 4	8	≥ 16

* The following antimicrobial breakpoints differ from current NCCLS recommendations from document M100 for organisms that grow aerobically: amikacin, minocycline, and sulfamethoxazole.

† Ciprofloxacin may be used as a class representative for the older fluoroquinolones: ciprofloxacin, ofloxacin, and levofloxacin. However, ciprofloxacin is not as active *in vitro* as the new 8-methoxyfluoroquinolones (i.e., gatifloxacin and moxifloxacin) and cannot be used as the class representative for these agents. See Section 5.3 and Summary of Comments question and response number 23.

‡ Class representative for newer macrolides.

§ Proposed breakpoint. No *Nocardia* isolates with linezolid MIC values >8 µg/mL have been reported (Brown-Elliott BA, Ward SC, Crist CJ, Mann LB, Wilson RW, Wallace RJ Jr: *In vitro* activities of linezolid against multiple *Nocardia* species. *Antimicrob Agents Chemother.* 2001;45:1295-1297).

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Appendix A. Case Scenarios for MTBC Drug Susceptibility Testing

These scenarios are intended as examples for guidance on algorithms for the performance of MTBC drug susceptibility testing with primary and secondary antituberculous drugs. The two panels represent proposed recommendations for a full panel and a panel with fewer primary drugs (referred to here as a reduced panel of primary drugs). Other considerations include the availability of referral testing and the amount of time required to provide results for primary drugs. The patient culture source may be a culture on solid media submitted by a referring hospital or a liquid primary culture in the laboratory. The recommendation is for primary drug susceptibility testing in a rapid broth method.

NOTE: Background information on initial drug resistance is provided for patient scenarios 1 through 4 based on data from U.S. National Surveillance System, Division of TB Elimination, CDC. Figures are based on analysis of drug susceptibility results reported for cases during the years 1993-1997 that had results for at least INH, RIF, EMB, and PZA. Approximately 90% of culture-positive cases had test results for the three first-line drugs; however, only 54% of these had results reported for PZA. Critical concentrations are not reported with drug susceptibility results sent to the national surveillance system. Reporting instructions indicate that any degree of resistance should be entered on the report to CDC.

Patient 1 (86% of initial isolates were susceptible to all four drugs.)

Results of full panel

Drug	Equivalent Reference Method Concentration	Result
INH	0.2 μg/mL	S
INH	1.0 μg/mL	S
RIF	1.0 μg/mL	S
EMB	5.0 μg/mL	S
PZA*	100 μg/mL	S

Next step: Report results. No further testing required. (Testing should be repeated if a positive culture is obtained after three months of treatment.)

Results of reduced panel

Drug	Equivalent Reference Method Concentration	Result
INH	0.2 μg/mL	S
RIF	1.0 μg/mL	S
EMB	5.0 μg/mL	S

Next step: See above.

* For equivalent reference concentration for PZA the Bactec 460TB is the reference method.

Appendix A. (Continued)

Considerations: 1) Overall, 1% of initial isolates were resistant to PZA alone. 2) There is a low probability of treatment failure with standard therapy and MTBC isolates resistant to PZA only.

Patient 2 (7% of initial isolates were resistant to INH and susceptible to RIF, EMB, and PZA.)

Results of full panel

Drug	Equivalent Reference Method Concentration	Result
INH	0.2 μg/mL	R
INH	1.0 μg/mL	S
RIF	1.0 μg/mL	S
EMB	5.0 μg/mL	S
PZA*	100 μg/mL	S

Next step: Report results with the comment: **These test results indicate low-level resistance to INH. Some experts believe that patients infected with strains exhibiting this level of INH resistance may benefit from continuing therapy with INH. A specialist in the treatment of tuberculosis should be consulted concerning the appropriate therapeutic regimen and dosages.**

For isolated low-level INH resistance, secondary drugs need not be tested unless drug intolerance is encountered.

Considerations: Confirm INH resistance by agar proportion method if the laboratory has a low volume of testing and rarely detects resistance, or if the laboratory has less experience with susceptibility testing using a rapid broth method. If both a rapid broth method and agar proportion are performed by the laboratory, it is good practice to routinely confirm resistance detected by the rapid broth method.

Results of reduced panel

Drug	Equivalent Reference Method Concentration	Result
INH	0.2 μg/mL	R
RIF	1.0 μg/mL	S
EMB	5.0 μg/mL	S

Next step: Report results with the comment: **This test result indicates the presence of at least low-level resistance to INH**. **Testing at a higher level of INH will be performed. A specialist in the treatment of tuberculosis should be consulted concerning the appropriate therapeutic regimen and dosages.**

Additional testing of PZA and high concentration of INH should be performed. Consideration should also be given to simultaneously testing a battery of secondary drugs.

* For equivalent reference concentration for PZA the Bactec 460TB is the reference method.

Appendix A. (Continued)

Considerations: 1) The probability of PZA resistance increases with the presence of INH resistance, and approximately 3.7% of initial isolates resistant to INH were resistant only to INH and PZA. 2) If resistant to the higher concentration of INH and/or resistant to PZA, there may also be a need for secondary drug testing. Referral laboratories should develop algorithms that avoid providing susceptibility testing in a "piecemeal" fashion. Confirm INH resistance as above.

Example: Results of reduced panel are available within 28 days of specimen receipt with rapid testing methods. Shipment to state or provincial laboratory, however, will require a pure culture. If further resistance is detected, then 21 days are required for secondary drug testing with the agar proportion method (28 days for primary, +14 days for solid culture [inoculated from a rapid broth system], +28 days to receive, set up, read, and report secondary drugs equals 2.5 months).

Patient 3 (7% of all initial isolates were resistant to INH and susceptible to RIF, EMB, and PZA.)

Results of full panel

Drug	Equivalent Reference Method Concentration	Result
INH	0.2 μg/mL	R
INH	1.0 μg/mL	R
RIF	1.0 μg/mL	S
EMB	5.0 μg/mL	S
PZA*	100 μg/mL	S

Next step: Report results with the comment: **These test results indicate high-level resistance to INH**. **A specialist in the treatment of tuberculosis should be consulted concerning the appropriate therapeutic regimen and dosages.**

Physicians may request secondary drugs if there is intolerance to a primary drug, or for prophylaxis of contacts (e.g., quinolone). Isolated INH resistance may be successfully treated with the three remaining drugs.

Results of reduced panel

Drug	Equivalent Reference Method Concentration	Result
INH	0.2 μg/mL	R
RIF	1.0 μg/mL	S
EMB	5.0 μg/mL	S

Next step: Report results with the comment: **This test result indicates the presence of at least low-level resistance to INH**. **Testing at a higher level of INH will be performed. A specialist in the treatment of tuberculosis should be consulted concerning the appropriate therapeutic regimen and dosages.**

* For equivalent reference concentration for PZA the Bactec 460TB is the reference method.

Appendix A. (Continued)

Additional testing of PZA and high concentration of INH should be performed. Consideration should also be given to simultaneously testing all secondary drugs.

Considerations: Same as for patient 2.

Patient 4 In 1997, only 1% of initial isolates from patients with drug susceptibility test results were resistant to INH and RIF.

Results of full panel

Drug	Equivalent Reference Method Concentration	Result
INH	0.2 μg/mL	R
INH	1.0 μg/mL	R
RIF	1.0 μg/mL	R
EMB	5.0 μg/mL	S
PZA*	100 μg/mL	S

Next step: Report results. **Because this is an example of multiple drug resistant tuberculosis (MDRTB), it is important to test all secondary drugs**. Confirm resistance by agar proportion method but do not delay reporting of rapid broth testing results.

Considerations: Based on the difficulties in treating MDRTB, there should be assurance that the referral laboratory offers all secondary drugs to avoid piecemeal provision of secondary drug results. This is especially true since shipment of a culture and testing secondary drugs in the agar proportion method will delay secondary drug results.

Results of reduced panel

Drug	Equivalent Reference Method Concentration	Result
INH	0.2 μg/mL	R
RIF	1.0 μg/mL	R
EMB	5.0 μg/mL	S

Next step: Report results. Additional testing of PZA and high concentration of INH. Perform full battery of secondary drugs. Confirm resistance in agar proportion method but do not delay reporting of rapid broth testing results.

Considerations: Same as above.

* For equivalent reference concentration for PZA the Bactec 460TB is the reference method.

Appendix A. (Continued)

Patient 5

Results of full panel

Drug	Equivalent Reference Method Concentration	Result
INH	0.2 μg/mL	R
INH	1.0 μg/mL	S
RIF	1.0 μg/mL	S
EMB	5.0 μg/mL	R
PZA*	100 μg/mL	S

Next step: Report results with the comment: **These test results indicate low-level resistance to INH and resistance to EMB**. **Some experts believe that patients infected with strains exhibiting this level of INH resistance may benefit from continuing therapy with INH. A specialist in the treatment of tuberculosis should be consulted concerning the appropriate therapeutic regimen and dosages.**

All secondary drugs should be tested. Confirm resistance by agar proportion method but do not delay reporting of rapid broth testing results. Confirmation by agar proportion should include additional higher concentration of EMB and testing of streptomycin.

Considerations: All secondary drugs should be tested when an isolate is resistant to any two primary drugs or RIF alone. Although there are problems with the reproducibility of EMB results, EMB resistance is usually found together with resistance to other drugs, so it is likely that these results will be confirmed. The panel of drugs in agar proportion should include additional concentrations of INH, streptomycin, and EMB.

Results of reduced panel

Drug	Equivalent Reference Method Concentration	Result
INH	0.2 μg/mL	R
RIF	1.0 μg/mL	S
EMB	5.0 μg/mL	R

Next step: Additional testing of PZA and high concentration of INH. All secondary drugs should be tested. Confirm resistance by agar proportion method but do not delay reporting of rapid broth testing results. Confirmation by agar proportion should include additional concentration for EMB and also testing for streptomycin.

Considerations: Same as above.

* For equivalent reference concentration for PZA the Bactec 460TB is the reference method.

Appendix A. (Continued)

Patient 6

Results of full panel

Drug	Equivalent Reference Method Concentration	Result
INH	0.2 μg/mL	S
INH	1.0 μg/mL	S
RIF	1.0 μg/mL	S
EMB	5.0 μg/mL	R
PZA*	100 μg/mL	S

Next step: Confirm EMB resistance by agar proportion method or repeat EMB test with rapid broth testing before reporting. Provide results for other drugs with EMB results "pending."

Considerations: True EMB mono-resistance is rare; therefore, it is very likely that this represents false resistance. Follow the manufacturer's instructions concerning EMB testing. True mono-resistance to EMB would not affect therapy (other than dropping EMB) for most patients.

Results of reduced panel

Drug	Equivalent Reference Method Concentration	Result
INH	0.2 μg/mL	S
RIF	1.0 μg/mL	S
EMB	5.0 μg/mL	R

Next step: Confirm EMB resistance by agar proportion method or repeat EMB test with rapid broth method before reporting. Consider additional testing of PZA.

Considerations: Same as above.

* For equivalent reference concentration for PZA the Bactec 460TB is the reference method.

Appendix B. McFarland 0.5 Barium Sulfate Turbidity Standard

To standardize the inoculum density, a $BaSO_4$ turbidity standard is used and may be prepared as follows:

(1) A 0.5-mL aliquot of 0.048 mol/L $BaCl_2$ (1.175% w/v $BaCl_2 \cdot 2H_2O$) is added to 99.5 mL of 0.18 mol/L (0.36 N) H_2SO_4 (1% v/v) with constant stirring to maintain a suspension.

(2) The correct density of the turbidity standard should be verified by using a spectrophotometer with a 1-cm light path and matched cuvettes to determine the absorbance. The absorbance at 625 nm should be 0.08 to 0.10 for the 0.5 McFarland standard.

(3) The barium sulfate suspension should be transferred in 4- to 6-mL aliquots into screw-cap tubes of the same size as those used for growing or diluting the bacterial inoculum.

(4) These tubes should be tightly sealed and stored in the dark at room temperature.

(5) The barium sulfate turbidity standard should be vigorously agitated on a vortex mixer before each use and inspected for a uniformly turbid appearance. If large particles appear, the standard should be replaced.

Latex particle suspensions should be mixed by inverting gently, not on a vortex mixer.

(6) Monthly replacement of the barium sulfate standards or verification of their densities should be considered.

Appendix C. Scenarios and Examples of Possible Reports

Example 1. A laboratory testing INH only at the low ("critical") concentration, using the reference agar proportion method, and the isolate in question shows no growth on the drug-containing quadrant and adequate growth on the drug-free quadrant:

Option A

Antimycobacterial Agent	Interpretation
INH	Susceptible

Option B

Antimycobacterial Agent	Concentration (μg/mL)	Method	% Resistance	Interpretation
INH	0.2	Agar Proportion	0	Susceptible

Example 2. A laboratory testing INH only at the low ("critical") concentration, using the reference agar proportion method, and the isolate in question shows 100 colonies on the drug-containing quadrant and 150 on the drug-free quadrant:

Option A

Antimycobacterial Agent	Interpretation
INH	Resistant (see Note)

Option B

Antimycobacterial Agent	Concentration (μg/mL)	Method	% Resistance	Interpretation
INH	0.2	Agar Proportion	67	Resistant (see Note)

NOTE: This test result indicates the presence of at least low-level resistance to INH. Testing at a higher level of INH will be performed. A specialist in the treatment of tuberculosis should be consulted concerning the appropriate therapeutic regimen and dosages.

Example 3. A laboratory testing INH only at the low concentration, using a rapid broth method, and the isolate is determined to be susceptible at that concentration:

Option A

Antimycobacterial Agent	Interpretation
INH	Susceptible

Option B

Antimycobacterial Agent	Equivalent Reference Concentration (μg/mL)	Interpretation
INH	0.2	Susceptible

Option C

Antimycobacterial Agent	Concentration (μg/mL)	Method	Equivalent Reference Concentration (μg/mL)	Interpretation
INH	0.1	BACTEC 460TB	0.2	Susceptible

Example 4. A laboratory testing INH only at the low concentration, using a rapid broth method, and the isolate is determined to be resistant at that concentration:

Option A

Antimycobacterial Agent	Interpretation
INH	Resistant (see Note)

Option B

Antimycobacterial Agent	Equivalent Reference Concentration (μg/mL)	Interpretation
INH	0.2	Resistant (see Note)

Option C

Antimycobacterial Agent	Concentration (μg/mL)	Method	Equivalent Reference Concentration (μg/mL)	Interpretation
INH	0.1	BACTEC 460TB	0.2	Resistant (see Note)

NOTE: This test result indicates the presence of at least low-level resistance to INH. Testing at a higher level of INH will be performed. A specialist in the treatment of tuberculosis should be consulted concerning the appropriate therapeutic regimen and dosages.

Example 5. A laboratory testing INH at both concentrations, using the reference agar proportion method, and the isolate in question shows 30 colonies on the drug-containing quadrant at the lower concentration, no colonies at the higher concentration, and 150 colonies on the drug-free quadrant:

Option A

Antimycobacterial Agent	Concentration (μg/mL)	Method	Interpretation
INH	0.2	Agar Proportion	Resistant
INH	1.0	Agar Proportion	Susceptible (see Note)

Option B

Antimycobacterial Agent	Concentration (μg/mL)	Method	% Resistance	Interpretation
INH	0.2	Agar Proportion	20	Resistant
INH	1.0	Agar Proportion	0	Susceptible (see Note)

NOTE: These test results indicate low-level resistance to INH. Some experts believe that patients infected with strains exhibiting this level of INH resistance may benefit from continuing therapy with INH. A specialist in the treatment of tuberculosis should be consulted concerning the appropriate therapeutic regimen and dosages.

Example 6. A laboratory testing INH at both concentrations, using a rapid broth method, and the isolate in question is determined to be resistant at the lower concentration but susceptible at the higher concentration:

Option A

Antimycobacterial Agent	Equivalent Reference Concentration (μg/mL)	Interpretation
INH	0.2	Resistant
INH	1.0	Susceptible (see Note)

Option B

Antimycobacterial Agent	Concentration (μg/mL)	Method	Equivalent Reference Concentration (μg/mL)	Interpretation
INH	0.1	BACTEC 460TB	0.2	Resistant
INH	0.4	BACTEC 460TB	1.0	Susceptible (see Note)

NOTE: These test results indicate low-level resistance to INH. Some experts believe that patients infected with strains exhibiting this level of INH resistance may benefit from continuing therapy with INH. A specialist in the treatment of tuberculosis should be consulted concerning the appropriate therapeutic regimen and dosages.

Example 7. A laboratory testing INH at both concentrations, using the reference agar proportion method, and the isolate in question shows 60 colonies on the drug-containing quadrant at the lower concentration, 30 colonies at the higher concentration, and 150 colonies on the drug-free quadrant:

Option A

Antimycobacterial Agent	Concentration (μg/mL)	Method	Interpretation
INH	0.2	Agar Proportion	Resistant
INH	1.0	Agar Proportion	Resistant (see Note)

Option B

Antimycobacterial Agent	Concentration (μg/mL)	Method	% Resistance	Interpretation
INH	0.2	Agar Proportion	40	Resistant
INH	1.0	Agar Proportion	20	Resistant (see Note)

NOTE: These test results indicate high-level resistance to INH. A specialist in the treatment of tuberculosis should be consulted concerning the appropriate therapeutic regimen and dosages.

Example 8. A laboratory testing INH at both concentrations, using a rapid broth method, and the isolate in question is determined to be resistant at both the lower and the higher concentrations:

Option A

Antimycobacterial Agent	Equivalent Reference Concentration (μg/mL)	Interpretation
INH	0.2	Resistant
INH	1.0	Resistant (see Note)

Option B

Antimycobacterial Agent	Concentration (μg/mL)	Method	Equivalent Reference Concentration (μg/mL)	Interpretation
INH	0.1	BACTEC 460TB	0.2	Resistant
INH	0.4	BACTEC 460TB	1.0	Resistant (see Note)

NOTE: These test results indicate high-level resistance to INH. A specialist in the treatment of tuberculosis should be consulted concerning the appropriate therapeutic regimen and dosages.

Appendix D. Drugs Available for Susceptibility Testing of MTBC Using Commercial Shorter Incubation Systems Cleared for Use by the U.S. FDA* and Their Equivalence in the Agar Proportion Method

Antimicrobial Agent	System and Concentration (μg/mL)			
	BACTEC 460TB	BACTEC MGIT 960	ESP II	7H10 Agar
Isoniazid	0.1	0.1	0.1	0.2
	0.4	0.4	0.4	1.0
Rifampin	2.0	1.0	1.0	1.0
Ethambutol hydrochloride	2.5	5.0	5.0	5.0
	7.5		8.0	10.0
Pyrazinamide	100	100	-	-
Streptomycin	2.0	1.0	-	2.0
	6.0	4.0	-	10.0

Key:

- not available or not recommended
BACTEC 460TB and BACTEC MGIT 960 = BD (Sparks, MD)
ESP II = Trek Diagnostics Systems (Westlake, OH)

*Cleared for use by the U.S. FDA at the time this document was completed.

Appendix E. Procedure for Alkalization of Radiometric 12B Medium

To alkalize the standard radiometric 12B medium, 0.3 mL of a 3% solution of tripotassium phosphate is added to 4.0 mL of the 12B broth in a vial. This solution may be prepared from pure K_3PO_4, taking into account the actual activity indicated by the manufacturer. For example, if the potency is 97%, 3.06 g of K_3PO_4 powder is dissolved in 100 mL of distilled water. The solution should be sterilized by filtration and then can be stored in small aliquots in tightly closed tubes, preferably in the refrigerator (room temperature is also acceptable), for up to six months.

Appendix F. Agar Disk Elution Method for Testing Slowly Growing Nontuberculous Mycobacteria

(From Stone MS, Wallace RJ Jr, Swenson JM, Thornsberry C, Christensen LA. Agar disk elution method for susceptibility testing of *Mycobacterium marinum* and *Mycobacterium fortuitum* complex to sulfonamides and antibiotics. *Antimicrob Agents Chemother*. 1983;24[4]; 486-493. Modified with permission from authors and the American Society for Microbiology.)

The agar disk elution method for testing the NTM described here is similar to the agar proportion method for testing MTBC as described in Section 3.2.3.1.1.

(1) Add 0.5 mL of OADC enrichment to the bottom of each well in a six-well culture plate. The six-well culture plate is recommended for optimal distribution of the drug throughout the media.

(2) See Tables 6 and 7 for a list of antimicrobial agents to be tested. Place the appropriate antimicrobial disks (as listed in the following table) to be tested in the bottom of the well so that the OADC soaks into the disks. One well should be left without antimicrobial disks as a growth control.

Agar Disk Elution of Slowly Growing NTM

Antimycobacterial Agent / Concentration (µg)		# Disks in 5 mL Media	Final Concentration in Well (µg/mL)
Rifampin	5	1	1
Ethambutol hydrochloride	25	1	5
Isoniazid	5	5	5
Streptomycin	50	1	10
Clarithromycin	15	5	15
Amikacin	30	2	12
Ciprofloxacin	5	2	2
Trimethoprim-sulfamethoxazole	23.75/1.25	2	0.5/9.5
Doxycycline	30	1	6
Minocycline	30	1	6

(3) Allow plates to stand for 15 minutes at room temperature for the drug to elute from the disks into the enrichment.

(4) Add 4.5 mL of melted and cooled cation-adjusted Mueller-Hinton agar or Middlebrook 7H10 agar to each well and gently swirl to mix with a wooden applicator stick making sure that the disks are centered.

(5) Allow the agar to solidify with lids ajar for 30 to 60 minutes in a laminar flow hood so that any accumulated moisture can evaporate. The susceptibility plates may then be used immediately or stored in sealed plastic bags for up to one week.

(6) Test samples of batches of plates for sterility as described in the procedure for the agar proportion method for testing MTBC (Section 3.2.3.1.1).

(7) Prepare a suspension of the test organism in broth or sterile water until it matches the 0.5 McFarland turbidity standard. Dilute this suspension 1:100 with broth or sterile water and inoculate the wells with 100 microliters of this suspension.

(8) Incubate isolates of *M. marinum* at 28 to 30 °C for 7 days and isolates of *M. kansasii* at 35 °C. With *M. kansasii*, if no growth is visible at 7 days then reincubate up to 14 days.

(9) Susceptibility is defined as no growth in the wells containing antibiotics and greater than or equal to 80% reduction in colony size with sulfonamides. Resistance is defined as growth greater than the final concentration of drug in the agar (see Tables 6 and 7).

(10) Results are reported as less than or equal to (susceptible) or greater than the final concentration (resistant) in the well.

(11) **NOTE:** Most drugs, including INH, clarithromycin, rifabutin, ciprofloxacin, and ethambutol have not been validated using this method by a published comparison with other methods. (Rifabutin disks are not commercially available.)

NCCLS consensus procedures include an appeals process that is described in detail in Section 9 of the Administrative Procedures. For further information, contact the Executive Offices or visit our website at www.nccls.org.

Summary of Comments and Subcommittee Responses

M24-T2: *Susceptibility Testing of Mycobacteria, Nocardiae, and Other Aerobic Actinomycetes; Tentative Standard—Second Edition*

General

1. I am writing to express my concern about the recommendation in M24-T2 to perform isoniazid susceptibility testing of *M. tuberculosis* at two different concentrations, specifically at a concentration of 1 mg/L. Testing INH at this higher concentration gives no clinically validated useful information, and may result in improper therapy except by those very few individuals who are true experts in the chemotherapy of tuberculosis.

   As an alternative, a general recommendation could be made that higher concentration INH susceptibility testing might be useful in cases of multidrug resistance, and that such isolates be referred to reference laboratories. Regardless of what final recommendation is made, the paucity of clinical data on this point should be stipulated in the document.

- **Text has been added to the Introduction to Section 3.1 and to Section 3.2.3.3.**

2. The document does not mention the requirement for reporting susceptibility testing results to local and/or state public health officials in addition to the submitter of the sample. Susceptibility testing results are now part of the TB case report form.

- **Text regarding reporting requirements has been added to the seventh paragraph of the Introduction to Section 3.1.**

3. With the reclassification of streptomycin as a secondary drug, the document should spell out the pros and cons for this decision, in relation to TB control activities.

- **This has been addressed in the eleventh paragraph of the Introduction to Section 3.1.**

4. Concentrations for streptomycin and ethambutol in the radiometric method: Although the responsibility for determining appropriate drug concentrations in the rapid radiometric assay belong with the manufacturer, comments about this problem should be included in this document. Standardization of the critical concentrations of streptomycin (SM) and ethambutol (EMB) to be used in the radiometric procedure has not been accomplished without controversy. It is noteworthy that the recommended concentrations for EMB and SM underwent adjustments over time.

- **The subcommittee acknowledges this issue. Please refer to the eleventh paragraph in the Introduction to Section 3.1 and Appendix A, patient 6.**

5. There is no clinical data to support the testing of two concentrations of ethambutol (and streptomycin), in contrast to isoniazid.

- **As stated, resolution remains to be determined when data become available.**

6. There is a need for the following to be addressed:

   a) Guidance in testing newer drug compounds

   b) Role of molecular assays

c) The use of molecular assays for susceptibility testing

- **This is beyond the scope of the document.**

7. *M. tuberculosis* complex isolates on which susceptibility testing was performed should be kept for at least one year in case additional characterization becomes necessary. This information should be included.

- **Text regarding holding of isolates has been added in the last paragraph of the Introduction to Section 3.1.**

Foreword

8. The World Health Organization has recently recommended the following terminology changes for the different types of resistance to antituberculosis drugs:

   a) Isolation of drug-resistant *Mycobacterium tuberculosis* from patients without a history of previous treatment should be referred to as "drug resistance among new cases" (instead of "primary resistance").

   b) Isolation of a drug-resistant strain from patients who have been treated for tuberculosis for at least one month should be referred to as "drug resistance among previously treated patients" (instead of "acquired resistance").

- **Text regarding the World Health Organization's recommended terminology has been added to the first paragraph of the Foreword.**

Standard Precautions

9. The guidelines only address "Standard Precautions," to be used with original specimens. It would be helpful to supply more guidance about biosafety issues when handling concentrated suspensions of *Mycobacterium tuberculosis* complex organisms.

- **References are provided in the Standard Precautions statement, which also refers to NCCLS document M29—*Protection of Laboratory Workers from Occupationally Acquired Infections*.**

Introduction

10. If patient care and public health are paramount, the step-wise provision of service can be severely compromised when the originating laboratory is slow in turnaround time. Since susceptibility-testing results are usually the last results obtained, additional delays can accumulate at each preceding step. Therefore, the turnaround time from the date a specimen has been taken until susceptibility test results are provided to the physician is an excellent marker to use to assess the laboratory services. The CDC recommends that for initial diagnostic specimens, mycobacteriology laboratories work toward the goal of reporting primary drug susceptibility results within 15 to 30 days (and not *average of 28 days*).

- **Text regarding specimen turnaround time has been added in the seventh paragraph of the Introduction to Section 3.1.**

11. It is important to emphasize that efforts to shorten turnaround times should not compromise the accuracy of the procedures. Based on laboratory findings of drug resistance, the patient's therapy may be altered to include more toxic and less effective compounds, and the duration of treatment may be extended. Therefore, it is very important that laboratory results are accurate.

- **The subcommittee agrees that this should be left to the discretion of each individual institution/laboratory.**

Section 3.2.2 (formerly Section 3.3)

12. These guidelines seem to be restricted to laboratories in North America. Additional comments about the use of egg-based media may be warranted, since this medium is in use globally and recommended by WHO and the International Union Against Tuberculosis and Lung Disease.

- **Text regarding media use has been added to Section 3.2.2.1.**

Section 3.2.3.1.1 (2) [formerly Section 3.4.1.1 (2)]

13. There is no reference for the preparation of the inoculum for the indirect proportion method.

- **The procedure recommended is the one utilized in most laboratories for several decades, and one that has been found to produce clinically useful results. It is essentially the procedure described in reference 6.**

Section 3.2.3.1.1 (6) [formerly Section 3.4.1.1 (6)]

14. Regarding the statement, "*…McFarland standards may be purchased commercially or prepared in-house using barium chloride and sulfuric acid…*": - there is no reference to the in-house preparation method described.

- **Text regarding McFarland standard preparation has been added.**

Section 3.2.3.1.1 (7) [formerly Section 3.4.1.1 (7)]

15. More emphasis should be placed on using an inoculum from broth. For example, when using the indirect method of specimen inoculation, an actively growing BACTEC culture is checked daily until the GI reading reaches 999 for two consecutive days. However, some strains, especially those that are drug-resistant, may grow poorly and peak before reaching a GI reading of 999. If that is the case, the vial is incubated one more day after peaking and then used as inoculum.

- **The subcommittee has addressed the inoculum concentration to be used with cultures grown in liquid medium [Section 3.2.3.1.1 (6)]. While the subcommittee members recognize that there may be problems in attaining an adequate concentration in liquid medium with some isolates, they agree that providing the procedural details to be used with specific commercial systems to assure an adequate inoculum concentration is beyond the scope of this document. Users of such commercial systems should consult the manufacturers' recommendations for the particular system utilized.**

Section 3.2.3.2.1 (7) [formerly Section 3.4.2.1 (8)]

16. *The plates should be protected from light during incubation*. Describe why this is necessary.

- **Text has been added as suggested.**

Section 3.2.3.3 (formerly Section 3.4.3)

17. A comment should be added suggesting that the following information should be provided in a susceptibility test report:

    - name, address, phone and fax number of the requestor;
    - patient's first and last name, date of birth, and county/state of residence;
    - specimen type and date taken;
    - test method and media used;
    - concentration of drug tested;
    - test results;
    - actual percentage resistance (agar proportion method)* the date the results were released; and where the results were reported and by what means.

    * Very important for therapeutic decisions

Overall, to make it more convenient for the healthcare provider and TB control officials, the reports should be in a cumulative format, with a line for comments and the present status of the assays.

- **The subcommittee has addressed some of these issues in Section 3.2.3.3. Different laboratory computer systems place varying constraints on the formats of laboratory reports and the types of patient data that can be incorporated into such reports. In addition, laboratories must contend with a variety of institutional and legal constraints on the types of information that can be included in laboratory reports. Therefore, the subcommittee members agreed that recommendations in this document should be confined to susceptibility results and their interpretations.**

Section 3.5.2 (formerly Section 3.6.2)

18. For routine quality control testing, each laboratory should establish the concentrations of the drugs to be used based on the level of susceptibility of a susceptible control strain. The highest concentration used should be the critical concentration of the drug, followed by at least three to four lower dilutions so that the control strain grows in the two lowest dilutions. Variations from such a pattern will quickly alert the laboratory to problems with the control strain or the drug concentrations.

- **Quality control has been addressed in Section 3.5.2.**

Section 4.2

19. In a patient who is intolerant of the macrolides or whose isolate has developed macrolide resistance, are there any additional agents other than the macrolides that could be tested for susceptibility with *Mycobacterium avium complex* (MAC)?

- **For some of the newer antimicrobial agents, no clinical data are yet available. Typically, patients with macrolide intolerance or disease due to macrolide-resistant MAC are treated with drug combinations without knowledge of their *in vitro* activity, especially in combination. However, until clinical data regarding the newer agents become available, testing ciprofloxacin, gatifloxacin, moxifloxacin, and/or linezolid may be reasonable. Using a broth microdilution method and established bacterial breakpoints, as described in NCCLS documents M7—*Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically* and M100—*Performance Standards for Antimicrobial Susceptibility Testing Supplemental Tables* is suggested for the newer agents except linezolid, which uses the breakpoints proposed for rapidly growing mycobacteria until further studies with MAC are available. The report should indicate that the breakpoints are tentative and not yet approved by NCCLS. The first-line antituberculous agents or clofazamine should not be tested, because it has been shown that results do not predict clinical outcome.**

Appendix

20. The document rightly stresses the need for more rapid turnaround times. However, the document suggests shipping a subculture from either a solid medium or broth medium to a referral laboratory for susceptibility testing. The document needs to address in more detail safe packaging and shipping considerations.

- **Shipping of medium has been addressed in the first paragraph in Appendix, A as suggested. Safety issues have been addressed on page xiii in the Standard Precautions statement.**

21. A comment should be added suggesting that before a laboratory decides to reduce the number of SIRE/PZA drugs to be tested, local TB controllers should be consulted. Patient management and surveillance are additional issues to be considered before reducing testing.

- **This has been addressed in the eleventh paragraph of the Introduction to Section 3.1.**

22. The case scenarios provided in the appendix suggest that long delays for generating susceptibility testing results are acceptable. In addition, it is stated that regional laboratories should develop algorithms that avoid providing susceptibility testing results in a "piecemeal" fashion, but the examples seem to suggest a piecemeal approach.

- **This has been addressed throughout Appendix A, as suggested.**

Section 5 (formerly Section 4.3)

23. Since the document states that ciprofloxacin should not be used as a class representative for quinolones to predict susceptibility of the nontuberculous mycobacteria (NTM) to the newer 8-methoxyfluoroquinolones, what breakpoints should be used if levofloxacin, gatifloxacin, or moxifloxacin is tested?

- **Ciprofloxacin can be used as the class representative for susceptibility testing of the NTM with levofloxacin and ofloxacin. However, because ciprofloxacin is not as active *in vitro* as the new 8-methoxyfluoroquinolones (i.e., gatifloxacin and moxifloxacin), individual testing of these agents is suggested if specifically requested, because the agent is being considered for clinical use. Although no laboratory or clinical studies have addressed appropriate breakpoints, use of the bacterial breakpoints, as described in NCCLS documents M7—*Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically* and M100—*Performance Standards for Antimicrobial Susceptibility Testing* for *Staphylococcus*, is tentatively suggested until such studies with the NTM are available. The report should indicate that the breakpoints are tentative and not yet approved by NCCLS.**

Delegate Comment and Subcommittee Response

M24-A: *Susceptibility Testing of Mycobacteria, Nocardiae, and Other Aerobic Actinomycetes; Approved Standard*

General

1. The information on Nocardia, especially breakpoints are not to my knowledge based on any M23 type investigations, but mainly uses Enterobacteriaceae guidelines. For an organism that grows much more slowly and is gram-positive, the Nocardia and other Actinomycetes should be removed from the M24 document.

- **NCCLS document M23 was developed as a guidance document for the Subcommittee on Antimicrobial Susceptibility Testing and applies only to regular aerobic bacteria, fastidious bacteria, and anaerobes. M23 does not apply to Nocardia, other aerobic actinomycetes, mycobacteria, or fungi.**

  The breakpoints for *Nocardia* and other aerobic actinomycetes are based on PK/PD data, organism population distributions, clinical data, breakpoints used for other organisms, and the experience of experts in the field. Currently, sufficient data exist to support susceptibility testing recommendations for MTBC and tentative recommendations for *Mycobacterium avium* complex, *Mycobacterium kanasii,* the rapidly growing mycobacteria (*Mycobacterium fortuitum* group, *Mycobacterium abscessus,* and *Mycobacterium chelonae*), and aerobic actinomycetes.

NOTES

The Quality System Approach

NCCLS subscribes to a quality system approach in the development of standards and guidelines, which facilitates project management; defines a document structure via a template; and provides a process to identify needed documents through a gap analysis. The approach is based on the model presented in the most current edition of NCCLS HS1—*A Quality System Model for Health Care.* The quality system approach applies a core set of "quality system essentials (QSEs)," basic to any organization, to all operations in any healthcare service's path of workflow. The QSEs provide the framework for delivery of any type of product or service, serving as a manager's guide. The quality system essentials (QSEs) are:

Documents & Records	Equipment	Information Management	Process Improvement
Organization	Purchasing & Inventory	Occurrence Management	Service & Satisfaction
Personnel	Process Control	Assessment	Facilities & Safety

M24-A addresses the quality system essentials (QSEs) indicated by an "X." For a description of the other NCCLS documents listed in the grid, please refer to the Related NCCLS Publications section on the following page.

Documents & Records	Organization	Personnel	Equipment	Purchasing & Inventory	Process Control	Information Management	Occurrence Management	Assessment	Process Improvement	Service & Satisfaction	Facilities & Safety
				M22	X M2 M7						M29

Adapted from NCCLS document HS1— *A Quality System Model for Health Care.*

Path of Workflow

A path of workflow is the description of the necessary steps to deliver the particular product or service that the organization or entity provides. For example, GP26-A2 defines a clinical laboratory path of workflow which consists of three sequential processes: preanalytical, analytical, and postanalytical. All clinical laboratories follow these processes to deliver the laboratory's services, namely quality laboratory information.

M24-A addresses the clinical laboratory path of workflow steps indicated by an "X." For a description of the other NCCLS documents listed in the grid, please refer to the Related NCCLS Publications section on the following page.

Preanalytic					**Analytic**		**Postanalytic**	
Patient Assessment	Test Request	Specimen Collection	Specimen Transport	Specimen Receipt	Testing Review	Laboratory Interpretation	Results Report	Post-test Specimen Management
					X M2 M7 M22	X M2 M7 M22	X M2 M7	X M2 M7

Adapted from NCCLS document HS1— *A Quality System Model for Health Care.*

Related NCCLS Publications*

M2-A8 **Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard—Eighth Edition (2003).** This standard contains currently recommended techniques for disk susceptibility testing, criteria for quality control testing, as well as providing updated tables for interpretive zone diameters.

M7-A6 **Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Sixth Edition (2003).** This standard describes reference methods for determination of minimal inhibitory concentrations (MICs) of aerobic bacteria by broth macrodilution, broth microdilution, and agar dilution, as well as providing updated tables for MIC interpretive criteria.

M22-A2 **Quality Assurance for Commercially Prepared Microbiological Culture Media; Approved Standard—Second Edition (1996).** This standard contains quality assurance procedures for manufacturers and users of prepared, ready-to-use microbiological culture media.

M29-A2 **Protection of Laboratory Workers from Occupationally Acquired Infections; Approved Guideline—Second Edition (2001).** Based on U.S. regulations, this document provides guidance on the risk of transmission of hepatitis viruses and human immunodeficiency viruses in any laboratory setting; specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials; and recommendations for the management of blood-borne exposure.

* Proposed- and tentative-level documents are being advanced through the NCCLS consensus process; therefore, readers should refer to the most recent editions.

NOTES

NOTES

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QSE Consulting
Quintiles, Inc.
Radiometer America, Inc.
Radiometer Medical A/S
Replidyne
Roche Diagnostics GmbH
Roche Diagnostics, Inc.
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SARL Laboratoire Carron (France)
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Sysmex Corporation (Japan)
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Xyletech Systems, Inc.
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YD Diagnostics (Seoul, Korea)

Trade Associations

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Associate Active Members

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Advocate Healthcare Lutheran General (IL)
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Albemarle Hospital (NC)
Allegheny General Hospital (PA)
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Antwerp University Hospital (Belgium)
Arkansas Department of Health
ARUP at University Hospital (UT)
Armed Forces Research Institute of Medical Science (APO, AP)
Associated Regional & University Pathologists (UT)
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BC Biomedical Laboratories (Surrey, BC, Canada)
Bermuda Hospitals Board
Bo Ali Hospital (Iran)
Brooks Air Force Base (TX)
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Cadham Provincial Laboratory
Calgary Laboratory Services
Cape Breton Healthcare Complex (Nova Scotia, Canada)
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Champlain Valley Physicians Hospital (NY)
Chang Gung Memorial Hospital (Taiwan)
Changi General Hospital (Singapore)
The Charlotte Hungerford Hospital (CT)
Children's Hospital (LA)
Children's Hospital (NE)
Children's Hospital & Clinics (MN)
Children's Hospital Medical Center (Akron, OH)
Children's Hospital of Philadelphia (PA)
Children's Medical Center of Dallas (TX)
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Clarian Health–Methodist Hospital (IN)
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CompuNet Clinical Laboratories (OH)
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Diagnosticos da América S/A (Brazil)
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Emory University Hospital (GA)
Fairview-University Medical Center (MN)
Federal Medical Center (MN)
Florida Hospital East Orlando
Focus Technologies (CA)
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Frye Regional Medical Center (NC)
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Indiana University
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Martin Luther King/Drew Medical Center (CA)
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MDS Metro Laboratory Services (Burnaby, BC, Canada)
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Medicare/Medicaid Certification, State of North Carolina
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Memorial Medical Center (LA) Jefferson Davis Hwy
Memorial Medical Center (LA) Napoleon Avenue
Mercy Medical Center (IA)
Methodist Hospital (TX)
MetroHealth Medical Center (OH)
Michigan Department of Community Health
Mississippi Baptist Medical Center
Monte Tabor – Centro Italo - Brazileiro de Promocao (Brazil)
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Montreal General Hospital (Canada)
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National University Hospital (Singapore)
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New England Fertility Institute (CT)
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New Mexico VA Health Care System
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North Shore – Long Island Jewish Health System Laboratories (NY)
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